Effects of exercise, with and without an associated energy deficit, on postprandial metabolism and appetite regulation

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ABSTRACT

In 1979, Donald Zilversmit published a landmark paper suggesting a role for postprandial metabolism in the development and progression of atherosclerosis, and in subsequent years, a number of postprandial metabolic perturbations have been highlighted as potential risk factors for atherosclerotic development. Interventions inducing favourable changes in postprandial metabolism may, therefore, have an important role in the management and control of cardiovascular disease and its associated risk factors. Acute exercise is one such intervention, successfully attenuating postprandial lipaemia and insulinaemia, enhancing endothelial function, reducing arterial stiffness and increasing postprandial fat oxidation. It also appears that exercise might facilitate a tighter coupling between energy intake and expenditure, mediating more stable and healthy body weights, perhaps by inducing changes in appetite, energy intake and circulating concentrations of 'hunger' hormones. It does, however, remain unclear as to what extent these changes are mediated by exercise *per se*, or the associated energy deficit and thus what effects exercise coupled with a state of energy balance would have on postprandial metabolism, appetite control and feeding behaviour. The aim of this thesis was, therefore, to investigate the immediate and delayed effects of exercise, coupled with a state of energy balance, on postprandial metabolism and appetite regulation.

Thirteen overweight, pre-menopausal women were recruited to compare the immediate effects of a state of energy balance coupled with high (exercise) or low (rest) energy turnover. Using a one-day model, each volunteer completed a 60-minute, moderate intensity treadmill walk prior to ingesting a test breakfast and 6-hour metabolic assessment period and a control trial during which no exercise was performed before breakfast. During the 6-hour observation period postprandial lipaemia, insulinaemia and glycaemia, total ghrelin and acylated ghrelin concentrations, pulse wave velocity and energy substrate utilisation were determined. Subjective ratings of appetite and food palatability and energy and macronutrient intakes at an ad libitum buffet meal were also recorded. Thirteen overweight and obese, middle-aged men were recruited to investigate the delayed effects of exercise with energy balance, using a two-day model, on postprandial metabolism and appetite control. On the afternoon of day-one, each volunteer completed one of three intervention trials; a moderate intensity treadmill walk inducing a net energy expenditure of 27 $kJ.kg^{-1}$.body mass, exactly the same walk with the net energy expenditure replaced or they performed no exercise. The following day, an 8.5-hour metabolic observation period was completed during which time postprandial lipaemia, insulinaemia and glycaemia,

leptin and total ghrelin concentrations, pulse wave velocity, energy substrate utilisation and subjective ratings of appetite were recorded in response to two separate oral fat tolerance tests.

The findings from these studies showed exercise, coupled with a state of energy balance, to have no significant effect, immediate or delayed, on postprandial lipaemia suggesting an exercise-induced energy deficit was required to lower postprandial triglyceride concentrations. A significant attenuation was, however, observed in postprandial insulin concentrations on the day following exercise with energy balance, indicative of enhanced insulin sensitivity. Both immediate and delayed increases in postprandial fat oxidation were observed following exercise, which led to lower (i.e. more negative) fat balances. This could potentially have important implications for the future management and regulation of the overweight and obese state. The role for exercise with energy balance in attenuating pulse wave velocity speeds remains equivocal with no immediate effect observed on postprandial central or peripheral pulse wave velocity, whilst significantly lower peripheral pulse wave velocity speeds were observed after a delay of some hours post-exercise. With regard to appetite regulation, exercise coupled with a state of energy balance does appear to reduce postprandial leptin concentrations on the following day, whilst total ghrelin remains resistant to any exercise-induced changes either immediately or some hours later. An exciting finding does, however, show acylated ghrelin concentrations to be significantly lower following exercise with energy balance, a response that may, to some extent, mediate subsequent changes in appetite. Changes in subjective ratings of appetite were transient, lasting just one to two hours after exercise with energy balance and energy and macronutrient intake at an *ad libitum* buffet meal consumed six hours after exercise with energy balance was not significantly altered.

The combined findings of this thesis suggest that exercise, coupled with a state of energy balance, induces favourable changes in postprandial insulinaemia, fat oxidation and peripheral pulse wave velocity, all of which may have important implications for lowering future risk of cardiovascular disease. Exercise performed in a state of energy balance may also contribute to the improved regulation of body weight, through effects on fat oxidation and balance and potentially via changes in 'hunger' hormones and appetite ratings, although the latter, in particular, requires further investigation.

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“Those who think they have not time for bodily exercise will sooner or later have to find time for illness”

Edward Stanley, Earl of Derby (1826-93)

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Unless otherwise indicated by acknowledgement or reference to published literature the work contained herein is that of the author.

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ABBREVIATIONS

TG – Triglyceride

NEFA – Non-esterified fatty acid

HDL – High density lipoprotein

VLDL – Very low density lipoprotein

LDL – Low density lipoprotein

ALP – Atherogenic lipoprotein phenotype

3-OHB – 3-hydroxybutyrate

PWV – Pulse wave velocity

ADMA – Asymmetrical dimethylarginine

NO – Nitric oxide

PFC – Prospective food consumption

BMI – Body mass index

BP – Blood pressure

$\dot{V}O_2$ max – Maximal oxygen uptake

RPE – Rate of perceive exertion

PAL – Physical activity level

RMR – Resting metabolic rate

CHO – Carbohydrate

VAS – Visual analogue scale

CHAPTER 1
INTRODUCTION

Rarely does a week go by without cardiovascular disease, obesity or diabetes being discussed in the media. This is perhaps unsurprising considering recent findings from the British Heart Foundation (Peterson *et al.*, 2005) showing cardiovascular disease (CVD) to be the leading cause of death for both men and women in the UK, killing more than 208,000 people every year. Furthermore, the proportion of men and women within the UK who are now overweight has reached levels of 67% and 59%, respectively, and 23% of men and 24% of women are obese. In addition to obesity, diabetes is becoming increasingly prevalent (Peterson *et al.*, 2005); in 2003 it was estimated that 4% of Britain suffered the disease, a figure likely to increase to just under 5% within the next 20 years, a percentage which equates to 2,141,000 people.

It is most likely no coincidence that during years in which the prevalence of CVD, obesity and diabetes has increased, the amount of time that people in the UK dedicate to physical activity has decreased (Peterson *et al.*, 2005). On average, only 36% of men and 26% of women meet the recommended level of 30 minutes of physical activity five or more times per week. This is a problem not only confined to adults; within England alone, data collected in 2002 suggested that 30% of boys and 39% of girls under the age of 15 were not regularly active. Considering that regular physical activity may help to protect against many diseases including CVD, obesity, insulin resistance, type 2 diabetes, dyslipidemia, hypertension, osteoporosis, some cancers and depression (Pedersen & Saltin, 2006), the implication of falling levels of physical activity within the UK is obvious and worrying. Increasing participation in physical activity is likely to be a successful and cost effective strategy for lowering the risk of CVD and its associated risk factors, and accordingly government targets for increasing participation in regular physical activity by 2022 in both adult and child populations have now been set both in England and Scotland (Peterson *et al.*, 2005).

Due to the extensive range of research questions addressed in this thesis, the following introductory chapter has been divided into two main sections. The first section primarily addresses postprandial metabolism whilst the second section of the chapter focuses on appetite regulation and feeding behaviour. Initially, the reader is introduced to CVD and its associated risk factors before discussing the metabolic pathways associated with fat and carbohydrate metabolism and how they interact. The rest of this section focuses on

metabolism in the postprandial state, addressing its associated implications for the development and progression of CVD and different research methods that can be used to investigate changes in the metabolic state. The first section concludes by investigating the effects of different exercise regimes on CVD and its associated risk factors with an emphasis on acute exercise and the role of an energy deficit and energy balance state.

The second section of this chapter introduces appetite and methods that can be used in research studies, both in the real world and within the laboratory, to investigate appetite and food intake. Regulatory mechanisms for appetite and feeding behaviour are discussed, including both physiological and behavioural mechanisms, and introducing appetite related hormones. Finally, as in the first section, the effects of exercise on appetite and food intake are discussed, whilst also considering potential problems exercise might have for appetite control.

1.1 Cardiovascular disease and associated risk factors

Cardiovascular disease describes a number of diseases affecting the heart and blood vessels, such as coronary heart disease, stroke, hypertension, with the common underlying cause of atherosclerosis (Hardman & Stensel, 2003). Hajjar and Nicholson (Hajjar & Nicholson, 1995) provide a detailed overview of the processes involved in the development of atherosclerosis and by doing so they identified two significant stages in its development; the origin of the lesion often described as a 'fatty streak' and the development of fibrous plaques. The continual development of these two processes inhibits normal functioning of a vessel and facilitates the occurrence of cardiac events and CVD. Atherosclerosis develops after the endothelial lining of a blood vessel becomes damaged, subsequently enabling molecules such as lipids, inflammatory and immune cells to cross the endothelium and enter the intimal layer where they are deposited. It is this stage that typically presents as the 'fatty streak' within a vessel wall. As atherosclerosis progresses, white blood cells such as monocytes migrate to the site of the lesion where after crossing the endothelial wall they form macrophages which digest lipid deposits (Hajjar & Nicholson, 1995). Within the lesion site there are also smooth muscle cells producing collagen and as smooth muscle cells and macrophages proliferate, they increase the overall size of the atheroma causing it to protrude into the lumen of the blood vessel. The development of a layer of connective tissue over the atheroma to retain the lipid and cell deposits forms a fibrous plaque (Hajjar & Nicholson, 1995). It is when the plaque becomes weakened and ruptures, releasing many thrombotic and coagulant factors into the

blood, that coronary thrombosis can develop promoting the onset of coronary events such as myocardial infarction (Hansson, 2005).

A number of factors might predispose an individual to a higher risk of suffering CVD, some of which are predetermined and cannot be modified e.g. gender, age, family history of CVD. Other factors however, such as poor diet, including excess fat and energy intakes and low fruit and vegetable consumption, smoking, blood pressure and physical activity, to name just a few, are within an individual's own control. To discuss all modifiable factors is beyond the scope of this thesis, but those risk factors addressed in subsequent experimental chapters, are described below.

1.1.1 Dyslipidaemia

Dyslipidemia describes an abnormal and undesirable circulating concentration of plasma lipids; three examples of which are elevated cholesterol, hypertriglyceridaemia and the atherogenic lipoprotein phenotype.

Cholesterol is a steroid involved in the formation of steroid hormones and bile salts and is also an important structural component of cell membranes (Hardman & Stensel, 2003). Cholesterol is hydrophobic and subsequently carried within the blood via specialised transporter particles called lipoproteins. Low density lipoproteins (LDL) transport a majority of cholesterol, moving it from sites of synthesis to other tissues, whilst high density lipoproteins (HDL) transport cholesterol away from tissues to the liver where it is broken down (Frayn, 2003). Although cholesterol is an integral component of metabolism, should the circulating concentration of cholesterol or cholesterol carrying lipoproteins become too high, it becomes an important risk factor for CVD. The accumulation of cholesterol within arterial walls of a blood vessel is a characteristic feature of atherosclerosis (Assmann & Nofer, 2003) and large population based studies have shown elevated total cholesterol concentrations to be associated with increased risk of morbidity and mortality from coronary disease (Kannel *et al.*, 1971;Martin *et al.*, 1987). As well as total cholesterol, the concentration of different sub-classes of cholesterol also has important implications for cardiovascular risk. Elevated LDL, especially smaller more dense particles which correlate with the degree of inter-media thickening in a blood vessel wall (Skoglund-Andersson *et al.*, 1999), are associated with an increased cardiovascular risk. A small LDL particle size has also been associated with a greater risk of developing ischemic heart disease (Lamarche *et al.*, 1997). High circulating concentrations of HDL cholesterol are inversely correlated with cardiovascular risk (Gordon *et al.*, 1989) and just

a small increase in HDL cholesterol has an important effect on the risk of heart disease (Hardman & Stensel, 2003). Conversely, low levels of HDL have been shown to predict the incidence of coronary heart disease in both men and women (Franceschini, 2001). Therefore, although total cholesterol concentrations are important, determining the subclass distribution of cholesterol provides a more meaningful and accurate insight into the risk of future CVD.

Triglycerides (TG) are the main source of fat consumed within the diet and both dietary and endogenous TG are excellent sources of energy (Frayn, 2003;Hardman & Stensel, 2003). Like cholesterol, TG is hydrophobic and relies upon lipoproteins for transport. Dietary TG is housed within chylomicron particles whilst endogenously derived TG is transported within very low density lipoproteins (VLDL) (Frayn, 2002). Also, similar to cholesterol, TG is essential for maintaining metabolic function but an elevated circulating concentration of TG, or hypertriglyceridaemia, is associated with an increased risk of CVD. A meta analysis of 17 population based studies (Austin *et al.*, 1998) revealed that for men, an increase in plasma TG concentrations of 1.0 $mmol.l^{-1}$ was associated with a relative CVD risk of 1.32, indicative of a 32% increase in the risk of CVD. In women, a 1.0 $mmol.l^{-1}$ increase in TG was associated with a relative risk of 1.76, indicative of a 76% increase in risk (Austin *et al.*, 1998).

A final dyslipidaemic state to be considered is the atherogenic lipoprotein phenotype; a preponderance of small dense LDL particles, a low concentration of HDL cholesterol and a high concentration of TG (Frayn, 2003). It has been suggested that the clinical importance of the atherogenic lipoprotein phenotype may be greater than that associated with cholesterol alone (Sattar *et al.*, 1998). The presence of the atherogenic lipoprotein phenotype has been associated with a two-to-four fold increase in cardiovascular risk (Austin *et al.*, 1998;Rizzo & Berneis, 2005) and a relatively high prevalence (26%) of the condition has been observed in patients with peripheral artery disease (Rizzo *et al.*, 2007) . Furthermore, the atherogenic lipoprotein phenotype is believed to be a significant contributory factor to the development of type 2 diabetes and may also increase coronary risk in those with pre diabetes (Superko, 1996).

1.1.2 Obesity

Obesity is a condition of increased fat storage and excess body weight, the cause of which is an energy intake greater than expenditure inducing a positive energy balance (Jequier, 2002). For a constant energy expenditure, excessive food and drink intake will promote

weight gain where as a stable energy intake coupled with excess physical activity will promote weight loss. The imbalance in energy intake and energy expenditure does not need to be large to induce changes in body weight; an energy intake of just 5% higher than energy expenditure can result in 5kg weight gain each year, which if repeated every year has obvious implications for the development of obesity (Jequier, 2002). It is believed that energy balance is more closely mediated by fat rather than carbohydrate (Flatt, 1995). An increase in dietary carbohydrate is typically met by an increase in carbohydrate oxidation, helping to maintain relatively constant carbohydrate storage. Unlike carbohydrate however, an increase in fat intake is not coupled with an increase in fat oxidation, thus greater fat storage ensues, promoting an overweight and obese state (Flatt, 1995). However, it should also be considered that an increase in carbohydrate oxidation could cause a reciprocal decrease in fat oxidation, thus also contributing to a positive fat balance and weight gain. Interventions, which increase fat oxidation and lower fat balance, will therefore have important implications for regulating body weight.

Currently no clear definition of obesity exists, however a common method used to categorise and classify an individuals weight status is to examine their body mass in relation to their height, achieved by calculating the body mass index or BMI. BMI is calculated by dividing an individuals mass (in kilograms) by their height (in metres) squared and **Table 1.1** shows the categories typically used to classify obesity, data based on those provided by the American College of Sports Medicine (Balady *et al.*, 2000).

Table 1.1 BMI categories used for the classification of obesity

	BMI ($kg.m^{-2}$)
Underweight	< 18.5
Normal weight	18.5 – 24.9
Overweight	25.0 – 29.9
Obese, class	
I	30.0 – 34.0
II	35.0 – 39.9
III	≥ 40.0

Obesity is a significant cardiovascular risk factor being associated with coronary heart disease (Haffner, 2006), increased arterial stiffness (Zebekakis *et al.*, 2005) and future development of diabetes (Haffner, 2006). As well as the total amount of fat stored within tissues, the location of fat storage is also important. Fat can be deposited in the upper

body, android obesity, or within the lower body, gynoid obesity and of the two, it is android obesity that is associated with greater health risks; lower HDL and elevated TG concentrations (Frayn, 2002) and the progression of atherosclerosis (Kortelainen & Sarkioja, 1999). In addition to android and gynoid fat deposition, there is visceral and subcutaneous fat storage. Accumulation of visceral fat is also associated with greater risk contributing to a hypertriglyceridaemic profile (Couillard *et al.*, 1998), glucose intolerance and insulin resistance (Yang & Smith, 2007), all of which increase an individual's risk of suffering future coronary heart disease. Using BMI to classify obesity does not take into consideration the location of the fat storage and subsequently the measurement of waist circumference and calculation of the waist to hip ratio have become useful tools for assessing weight status and its associated risk for CVD (Despres *et al.*, 2001). A waist to hip ratio above 0.87 has been associated with the development of coronary arterial lesions (Kortelainen & Sarkioja, 1999) and elevated waist to hip ratios are associated with an increased risk of total mortality and mortality from coronary heart disease (Katzmarzyk *et al.*, 2006).

1.1.3 Insulin resistance and type 2 diabetes

Insulin resistance has been described as the "requirement for greater concentrations of insulin than normal to elicit a given metabolic response" (Frayn, 2002). With regard to glucose control, this means that for a particular concentration of insulin in the blood, less glucose is cleared or alternatively, a higher concentration of insulin is required to clear the same amount of glucose. Four progressive stages of insulin resistance have been identified, firstly, a state of hyperinsulinaemia in response to food; secondly a state of hyperinsulinaemia and hyperglycaemia in response to food, thirdly, elevated fasting insulin and glucose levels and finally the development of type 2 diabetes (Kendrick, 2003). It is not however inevitable that once an individual shows the initial symptoms of insulin resistance they will further progress through all four stages. If treated, insulin resistance can be successfully reversed. The development of type 2 diabetes most frequently manifests itself in the later years of life, although its prevalence among young children and adolescents is increasing (Lazar, 2005). Type 2 diabetes is characterised by the resistance of body tissues to the effects of insulin and unlike type 1 diabetes, the pancreas is initially able to secrete insulin effectively. The constant demand for high concentrations of insulin can eventually, however, cause β cell failure facilitating the transition from insulin resistance to diabetes. Typical treatment strategies for type 2 diabetes therefore tend to focus on dietary and lifestyle interventions, although treatment with insulin sensitizing drugs (Gillies *et al.*, 2007) and or insulin may be used in the later stages of the disease

(Garber *et al.,* 2007).

Individual features of type 2 diabetes, including insulin resistance, hyperglycaemia and hyperinsulinemia, and type 2 diabetes itself are strong and independent risk factors for CVD. The risk of both total and CVD mortality is almost doubled in people with hyperglycaemia ≥ 11.1 mmol.l^{-1}, measured in response to an oral glucose load (Shaw *et al.*, 1999). The incidence of CVD also increases with increasing insulin resistance (Hanley *et al.*, 2002). The San Antonio Heart Study reported patients with insulin resistance to suffer a larger number of cardiovascular risk factors, including elevated TG and blood pressure and lower HDL, compared with healthy non-diabetic individuals (Haffner *et al.*, 2000). Furthermore, insulin resistance is believed to be a key factor mediating the progression of endothelial dysfunction (Lteif *et al.*, 2005). With regard to type 2 diabetes itself, the disease is associated with an increased risk of coronary heart disease (Haffner *et al.*, 1998) and worryingly CVD has been suggested to account for up to 70% of the deaths reported in people with type 2 diabetes (Feher *et al.*, 1999).

1.1.4 The metabolic syndrome

The metabolic syndrome describes a clustering of factors all of which increase an individual's risk of suffering CVD (Ford, 2004). A number of organisations provide their own diagnostic criteria, however it is those published by the International Diabetes Federation (IDF) (Alberti *et al.*, 2005) that will be referred to in this thesis. The metabolic syndrome is diagnosed if an individual presents with at least three of the following criteria:

- Central obesity: Waist circumference ≥ 94 cm for European men and ≥ 80 cm for European women*

Plus any two of the following factors:

- High TG levels: ≥ 1.7 mmol.l^{-1} or treatment for this lipid abnormality
- Low HDL: < 1.03 mmol.l^{-1} in men and < 1.29 mmol.l^{-1} in women or treatment for this lipid abnormality
- High blood pressure: Systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg, or treatment for hypertension
- High fasting plasma glucose: ≥ 5.6 mmol.l^{-1} or diagnosed with type 2 diabetes

*Separate criteria are suggested for identifying central obesity in men and women from different ethnic backgrounds (Alberti *et al.*, 2005).

The metabolic syndrome is associated with an increased risk of suffering fatal and non-fatal CVD (Dekker *et al.*, 2005;Wang *et al.*, 2007) and mortality from coronary heart disease (Wang *et al.*, 2007). Furthermore, the higher the number of the metabolic syndrome criteria an individual suffers, the greater their risk of CVD (Dekker *et al.*, 2005). The clinical relevance of the metabolic syndrome has however been questioned (Chew *et al.*, 2006) as it is unclear whether it has any added value in predicting an individual's risk of suffering CVD above that of the individual diagnostic criteria (Kahn *et al.*, 2005). The use of so many definitions for the metabolic syndrome also has its limitations as the identification of people at risk from CVD varies according to the diagnostic criteria used (Kahn *et al.*, 2005). Despite these concerns however, the metabolic syndrome does provide a useful tool for medical practitioners, and researchers, to identify individuals with an elevated risk of CVD who are therefore likely to benefit from future lifestyle changes including dietary and physical activity interventions (Grundy *et al.*, 2005).

1.2 Metabolism in the postabsorptive state

Postabsorptive refers to a state in which most of the food from the previous meal has been fully absorbed but not much more time has passed (Frayn, 2003), *e.g.* after an overnight fast prior to any food being consumed. It is in this state that experimental procedures implemented in the following chapters commenced and therefore the following section will consider the metabolic processes involved in both lipid and carbohydrate metabolism during the postabsorptive state.

Glucose is maintained at fairly stable concentrations within the blood, usually around 5.0 $mmol.l^{-1}$. Entry of glucose into the blood is via absorption from the intestine, breakdown of glycogen stores in the liver or from gluconeogenesis whilst the extraction of glucose is predominantly mediated by its uptake into body tissues (Frayn, 2003). This process of glucose production and removal from the blood is under tight hormonal control, particularly by the action of insulin and glucagon. Both insulin and glucagon are pancreatic hormones, glucagon is secreted in order to elevate plasma glucose concentrations, whilst the main function of insulin is to lower plasma glucose concentrations. In the postabsorptive state, there is little glucose available from absorption at the intestine but due to its continual utilisation within tissues such as the brain, the concentration of glucose in the blood falls slightly causing a shift in the hormonal balance to favour glucagon (Klover & Mooney, 2004). In this situation where no exogenous source of glucose is available, a majority of glucose is liberated from the liver via glycogenolysis and gluconeogenesis (Klover & Mooney, 2004). The extent to which each

of these processes contributes to the release of glucose varies depending on glycogen stored from the previous meal and the hormonal balance, elevated glucagon concentrations favour gluconeogenesis over glycogenolysis, although on average a majority of glucose is provided by glycogenolysis (Frayn, 2003;Klover & Mooney, 2004). Glycogenolysis is the process in which stored glycogen is broken down and converted to glucose. Glycogen phosphorylase acts upon and breaks down glycogen, releasing glucose-1-phosphate which can be converted to glucose and released into the circulation. The activity of glycogen phosphorylase is augmented by glucagon, adrenaline and noradrenaline and also by glucose itself as the more glucose available to the liver the greater the activity levels are (Frayn, 2003). Gluconeogenesis is the formation of glucose within the liver from sources other than glycogen, namely alanine, glycerol and lactate (Felber & Golay, 1995). Similar to glycogenolysis, gluconeogenesis is stimulated by glucagon and inhibited by insulin although further stimulation occurs when the supply of substrates is increased (Felber & Golay, 1995) for example when lactate is elevated following exercise.

Whereas glucose takes just one form within the blood, lipids circulate as fatty acids or TG particles and unlike glucose their concentration in the blood can vary dramatically. Non-esterified fatty acids (NEFA) are transported either bound to albumin already in plasma or as unbound particles. They enter the circulation from adipose tissue, a process mediated by the activity of hormone sensitive lipase (Frayn, 1998), whereas the rate at which NEFA are removed from the blood and utilised by tissues is very much determined by their circulating concentrations (Frayn, 2003). Unlike NEFA, and due to their hydrophobic properties, TG are housed within lipoproteins; exogenous TG transported in chylomicrons whilst endogenous TG is housed within VLDL (Karpe, 1999). In the postabsorptive state chylomicron TG concentrations will usually be negigible due to the absence of any dietary fat. Hormone sensitive lipase is inhibited by insulin and the decline in insulin in the postabsorptive state subsequently increases its activity, liberating greater amounts of NEFA from adipose (Frayn, 1998). These NEFA entering the circulation are transported into the liver and skeletal muscle via fatty acid transporter proteins (Gimeno, 2007). Within the liver, fatty acids may be oxidised, producing ketone bodies, which enter into the bloodstream, or alternatively, they may be re-esterified to form TG. Hepatic TG in combination with a single B100 apolipoprotein and other C and E proteins and cholesteryl ester are incorporated into VLDL particles and secreted from the liver into the circulation (Frayn, 2003). Here, their TG content is delivered to adipose tissue and muscle via lipolysis, a process mediated by lipoprotein lipase. A single VLDL particle can undergo several cycles of lipolysis producing a small, TG depleted particle which may either be

removed at the liver via the LDL receptor, or remain in the circulation to form LDL (Karpe, 1999). Within skeletal muscle, fatty acids are more likely directed towards oxidation rather than re-esterification (Felber & Golay, 1995) and in the postabsorptive state the utilisation of intramuscular TG is also increased with up to 50% of lipid oxidised in the post-absorptive state attributed to intramuscular TG (Felber & Golay, 1995).

Thus in the postabsorptive state, lower glucose concentrations and subsequently lower insulin and higher glucagon levels favour a situation in which the demands for glucose synthesis are met by glycogenolysis and gluconeogenesis. A greater liberation of fatty acids from adipose tissue, favoured by lower insulin concentrations, which are subsequently taken up by skeletal muscle and the liver and either oxidised or incorporated within VLDL ensures a continual source of energy on which the body can rely.

1.3 Metabolism in the postprandial state

The consumption of a meal and the arrival of dietary carbohydrate and fat move the body from a postabsorptive to postprandial state, a situation in which the body switches from being a system predominantly focused on producing substrate to a system promoting storage (Frayn, 2003).

Exogenous glucose enters the circulation raising blood glucose concentrations above baseline values, usually peaking around one hour after a meal (Frayn *et al.*, 1993). The magnitude of this glucose peak will depend on the amount of carbohydrate consumed and the composition of such carbohydrate (Lineback, 2005) and in response to this rise in glucose, insulin is released by the pancreas, raising the ratio of insulin to glucagon within the circulation. Such a shift in this hormonal balance has a number of consequences for metabolism in different tissues.

As mentioned above, the liver is the major source of glucose production in the postabsorptive state, however, following a meal the liver switches to glucose storage rather than production. Elevated concentrations of blood glucose arrive at the liver where, via GLUT-2 transporters, glucose is taken up into hepatocytes (Klover & Mooney, 2004). Increased glucose, coupled with elevated insulin, promotes glycogen synthesis via the activation of glycogen synthase and the inhibition of glycogen phosphorylase, the enzyme mediating the breakdown of glycogen to release glucose (Felber & Golay, 1995). During this time of glycogen storage, gluconeogenesis continues due to a constant supply of substrate such as lactate, however, in the postprandial state, glucose release is inhibited and

rather lactate is converted to glucose-6-phosphate which is then converted to glycogen for storage (Felber & Golay, 1995). Elevated glucose concentrations and the concomitant rise in plasma insulin also affects metabolism within other tissues, namely skeletal muscle and adipose tissue, tissues in which glucose uptake is mediated by GLUT-4 transporters (Klover & Mooney, 2004). Within skeletal muscle, glucose may be oxidised to provide energy and in fact in the postprandial state, muscle does appear to favour glucose to fatty acids as a fuel source (Frayn, 1998), an effect likely mediated by elevated glucose concentrations and attenuated NEFA. Some glucose may also be directed towards glycogen storage within the muscle as similar to the liver, insulin upregulates glycogen synthase activity (Felber & Golay, 1995). Within adipose tissue, glucose will be anaerobically metabolised, releasing lactate into the blood which as previously suggested is an important precursor for gluconeogenesis (Felber & Golay, 1995).

An increase in circulating insulin concentrations, as well as regulating glucose, also has implications for lipid metabolism. Elevated insulin dramatically, although not completely, inhibits the activity of hormone sensitive lipase and subsequently the release of NEFA into the circulation, thus a fall in blood NEFA concentrations is observed during the hours after a meal (Gill *et al.*, 2004). Such a drop in NEFA will restrict its use as a fuel source by tissues, especially skeletal muscle, and therefore facilitate the switch to glucose utilisation described above. Hepatic metabolism of NEFA also changes postprandially with the attenuated oxidation of fatty acids and formation of ketone bodies (Frayn, 2003). In contrast to the postabsorptive state, the importance of TG in postprandial metabolism is much greater. Exogenous TG is formed within the cells of the intestine wall and coupled with cholesteryl ester, phospholipid, apolipoprotein A1 and a single B48 apolipoprotein before being secreted as a chylomicron particle, entering the circulation via the thoracic duct (Cohn, 1998). Once within the circulation the newly formed chylomicron particle acquires additional structures, such as apolipoprotein CII, transforming them into viable particles for lipolysis via lipoprotein lipase. The continual hydrolysis of TG within chylomicrons produces a small remnant particle, eventually removed from the circulation at the liver via the LDL receptor related protein (Cohn, 1998).

Unlike glucose which rapidly enters the blood stream, the formation and secretion of chylomicron particles is a slower process and TG concentrations do not usually peak until four or so hours after a meal (Gill & Hardman, 2000). The clearance of TG from the circulation occurs predominantly at adipose tissue and skeletal muscle. Lipoprotein lipase situated on the cell membrane of adipose tissue lipolyse TG, liberating fatty acids, a

process stimulated by insulin. A majority of the liberated fatty acids will be taken up into adipose tissue to form TG, however some may 'spill over' back into the circulation remaining as NEFA particles (Frayn, 1998). Lipoprotein lipase is also situated within skeletal muscle membranes although unlike adipose tissue, the form of the enzyme expressed in muscle is suppressed by elevated insulin concentrations and the postprandial uptake of fatty acids into muscle is therefore reduced compared to adipose tissue (Frayn, 2003). In muscle fatty acids are oxidised to provide energy or esterified to form TG which is stored. As well as chylomicrons, postprandial TG also enter the circulation via hepatic secretion of VLDL. Chylomicrons and VLDL compete for lipolysis by lipoprotein lipase (Brunzell *et al.*, 1973) and with chylomicrons being the preferred substrate, during the hours following a meal there is typically a rise in plasma VLDL concentrations (Gill *et al.*, 2001a). In an attempt to attenuate a build up in VLDL TG there is a reduction in hepatic secretion of VLDL, mediated by elevated insulin concentrations (Karpe, 1999) and also the lower circulating NEFA concentrations which restricts their uptake into hepatocytes for secretion as VLDL (Frayn, 2003), but despite this regulatory mechanism, a majority of postprandial lipoproteins are attributed to a rise in VLDL particles.

At this point, the reader should consider however that the metabolic pathways described above are typical responses following a single meal. Plasma glucose and insulin concentrations will usually return to baseline within three to four hours after a meal (Frayn *et al.*, 1993) and a return of TG to baseline values is typically seen within eight hours (Gill *et al.*, 2004). In an every day situation however, these metabolic pathways are affected by the arrival of another meal or snack just a short time after the initial food was eaten. In this situation, a repeated rise in glucose and insulin concentrations, perhaps before a return to baseline values, will further promote the storage of glycogen within the liver and skeletal muscle. Furthermore, postprandial lipaemia is augmented by the arrival of more dietary fat and in a normal day, TG concentrations may become increasingly elevated above baseline as each meal is consumed. It is therefore important that investigations into postprandial metabolism consider the effect of multiple meals to provide a more detailed and accurate insight into metabolic regulation in the postprandial state.

1.4 Implications of the postprandial state for disease

In 1979, Donald Zilversmit produced a landmark research paper describing atherosclerosis as a 'postprandial phenomenon' (Zilversmit, 1979). Zilversmit introduced the notion that postprandial chylomicron particles and their remnants facilitated the development and progression of atherosclerosis. He did this using a series of studies and experiments

performed in both animal and human models. In rabbits with atherosclerosis, it was reported that the extent of hypercholesterolemia they suffered was primarily attributed to elevated chylomicron remnant concentrations. Also in rabbits, chylomicron remnant particles were reported to be as atherogenic as endogenously produced LDL and VLDL particles. In humans, specifically patients with type III hyperlipoproteinaemia, a high level of chylomicron remnant particles has been observed. The complete evidence for an atherogenic role of chylomicron remnants provided by Zilversmit, is too extensive to fully discuss here and the reader is therefore referred back to the full paper for a complete overview (Zilversmit, 1979). Since this landmark paper however, and as research has progressed, it has become evident that changes in pathways other than lipid metabolism also have implications for disease progression, further highlighting a detrimental role for the postprandial state in CVD.

1.4.1 Postprandial lipaemia

Postprandial lipaemia occurs after consuming a meal containing fat where there is a short term increase in the circulating concentrations of plasma TG lasting for up to eight hours in normal, healthy individuals (Cohn, 1998;Malkova & Gill, 2006). This increase in postprandial TG concentrations is associated with other potentially atherogenic perturbations (Cohn, 1998).

Following lipolysis of dietary TG by lipoprotein lipase, a smaller TG depleted chylomicron remnant particle remains in the circulation (Karpe, 1999). These remnant particles may be of greater risk for atherosclerosis than larger chylomicrons, as the larger particles formed initially after a meal are believed to be too large to permeate the arterial wall (Cohn, 1998); research has also shown postprandial concentrations of small chylomicron remnant particles to correlate with five-year progression of coronary atherosclerosis (Karpe *et al.*, 1994). The risk associated with chylomicrons in relation to VLDL remains unclear; patients with coronary artery disease appear to preferentially accumulate chylomicrons and their remnants and not VLDL compared to healthy controls (Simons *et al.*, 1987), although such findings remain equivocal (Karpe *et al.*, 1999) and high VLDL concentrations have been reported in patients with the metabolic syndrome and type 2 diabetes (Adiels *et al.*, 2005;Chan *et al.*, 2006). The lipolysis of postprandial VLDL-TG produces smaller VLDL remnant particles (Karpe, 1999), although the relevance of these for atherosclerotic progression has not been well established. There is evidence for the presence of VLDL within atherosclerotic plaques (Rapp *et al.*, 1994) and a high prevalence of apolipoprotein

B100, rather than apolipoprotein B48, remnant particles has recently been recorded in patients who suffered fatal cardiac mortality (Nakajima *et al.*, 2007).

A postprandial rise in TG also has implications for lower HDL (Cohn, 1998;Karpe, 1999) for two reasons. Firstly, impaired lipolysis of VLDL and chylomicron particles restricts the availability of surface structures such as phospholipids, apolipoprotein C and free cholesterol, subsequently reducing the rate at which HDL is formed (Cohn, 1998). Secondly, increased transfer of TG from VLDL and chylomicrons to HDL with movement of cholesteryl ester in the opposite direction also contributes to lower HDL; cholesteryl ester transfer proteins (CETP) mediate the transfer of cholesteryl ester from HDL (Karpe, 1999) and their activity is thought to be elevated in the fed state (Durlach *et al.*, 1996). The presence of a large number of VLDL and chylomicron particles in the postprandial state may also facilitate this transfer process as they offer a larger number of receptor particles into which cholesteryl ester can be deposited (Karpe, 1999). The movement of TG and cholesteryl ester between lipoprotein particles ultimately produces HDL particles rich in TG, which are lipolysed by hepatic lipase, producing a small, dense and less atheroprotective HDL_3 particle (Morgan *et al.*, 2004).

As well as HDL, high postprandial TG concentrations also affect the metabolism of LDL. Similar to HDL, LDL lose cholesteryl ester to VLDL and chylomicrons, a process again mediated by CETP, whilst accumulating TG, producing TG rich LDL particles. Via the activity of hepatic lipase, which hydrolyses the TG core, a smaller, denser and lipid depleted LDL particle in produced. These particles are associated with increased atherosclerotic risk due to their size as they are more likely to leave the circulation and permeate the endothelial lining of a blood vessel (Krauss, 1998). Furthermore, once within the endothelium, the LDL particle is particularly susceptible to oxidative stress and these now oxidatively damaged LDL can be taken up into macrophages where the process of foam cell formation and early atherosclerosis begins (Hajjar & Nicholson, 1995).

1.4.2 Postprandial hyperglycaemia and hyperinsulinaemia

Hyperglycaemia describes an elevated concentration of glucose within the blood, usually characterized by increased blood glucose two hours after an oral glucose tolerance test or mixed meal (Ceriello, 2004). It is these postprandial 'spikes' in glucose, rather than fasting values, that are suggested to contribute to the development of CVD (Ceriello, 2004;Hanefeld *et al.*, 2000). A number of mechanisms have been proposed by which postprandial elevations in plasma glucose might mediate atherosclerosis. Acute glycaemia

can impede endothelial function, an early feature of atherosclerosis which might be a consequence of inhibited vasodilatation or because of a reduction in the synthesis or availability of nitric oxide, a known vasodilator (Ceriello, 2004). Postprandial hyperglycaemia may also promote a thrombotic state and increase inflammatory responses; increased platelet aggregation and circulating concentrations of Factor VII have been observed in response to hyperglycaemia (Miller, 1998). Furthermore hyperglycaemia reportedly stimulates an increase in intracellular adhesion molecule-1, which can facilitate the adhesion of monocytes to the endothelium of a blood vessel (Boyle, 2005). Although the exact mechanism by which hyperglycaemia induces such effects has not been elucidated, its role in increasing the production of free radicals may be important (Ceriello, 2004).

Postprandial hyperinsulinaemia is a delayed, augmented increase in plasma insulin concentrations following the consumption of a mixed meal; an effect likely caused by an increase in β-cell mass and or β-cell dysfunction (Kopp, 2003). Although the association between postprandial hyperinsulinaemia and CVD is not well established, postprandial hyperinsulinaemia has been associated with the incidence of coronary artery disease (Baltali *et al.*, 2003). Acute hyperinsulinaemia may also contribute to a delayed accumulation of TG rich lipoproteins, especially the intestinally derived chylomicron particles, perhaps due to a delayed clearance of exogenously derived TG rich particles. The effect of hyperinsulinaemia on hepatically derived VLDL particles does, however, appear more negligible (Harbis *et al.*, 2001). Repeated episodes of postprandial hyperinsulinaemia have also been implicated in the development and progression of insulin resistance and obesity (Kopp, 2003). The underlying mechanism for why hyperinsulinaemia promotes obesity has yet to be clarified but likely involves the role of insulin in promoting fat storage within adipose tissue by increasing lipoprotein lipase activity and decreasing hormone sensitive lipase activity (Frayn, 1998), possibly restricting fat oxidation in favour of carbohydrate utilisation (Flatt, 1995;Frayn, 2003) and impaired lipolysis (Large & Arner, 1998).

1.4.3 Impaired vascular function and a pro-thrombotic state

Metabolic perturbations occurring in the postprandial state might also contribute to endothelial dysfunction. Flow mediated dilation, a measure of endothelium-dependant relaxation, is impaired following an oral glucose tolerance test, an effect which appears independent of insulin (de Koning & Rabelink, 2002). Similar results have been seen following oral fat tolerance tests where endothelium-dependant dilation (Gaenzer *et al.*,

2001) and flow mediated dilation are maximally impaired within the three to four hours after a meal is consumed (de Koning & Rabelink, 2002;Vogel *et al.*, 1997). The effect of glucose on the endothelium is via its role in increasing oxidative stress and lessening the effectiveness of antioxidant defence systems. With regard to the effect of lipoproteins on endothelial function, remnant chylomicron and VLDL particles have been shown to impair endothelium dependant vasodilation (Doi *et al.*, 1998) as have small, dense, oxidised LDL particles (Hein *et al.*, 2000). The mechanism for such an effect is again likely to involve the increased oxidative stress associated with a high fat meal and indeed the prescription of antioxidants has been shown to enhance postprandial endothelial function in both healthy (Neri *et al.*, 2005) and diseased (Anderson *et al.*, 2006) populations. Although any postprandial impairments in endothelial function do appear to be reversed within a few hours, in a typical situation where an individual eats meals and snacks at regular intervals throughout the day, the continued exposure of the endothelium to oxidative stress will potentially have detrimental effects on atherosclerotic progression (de Koning & Rabelink, 2002).

Inflammatory markers such as C-reactive protein, leucocyte concentrations, interleukins and compliment 3 (Alipour *et al.*, 2007;Kanda & Takahashi, 2004) have all been related to CVD. Following high fat, high carbohydrate and mixed composition test meals, neutrophil recruitment and the concentration of interleukin 8 and interleukin 6 have been shown to increase, changes which may mediate a reduction in endothelial function (Lundman *et al.*, 2007;van Oostrom *et al.*, 2003). Postprandial lipaemia has also been associated with increased activation of leucocytes in patients with atherosclerosis and healthy volunteers, an activation which may be stimulated to some degree by an interaction with chylomicrons and chylomicron remnants (Alipour *et al.*, 2007). Compliment 3, another valuable marker of inflammation which has been found within atherosclerotic plaques is directly stimulated by chylomicrons and as such provides another mechanism by which postprandial lipaemia may mediate a proinflammatory state (Alipour *et al.*, 2007).

The formation of thrombi and the deposition of fibrin are both factors which may promote and accelerate the development of atherosclerotic lesions within a blood vessel. Factor VII coagulant activity (FVIIc) is a strong predictor of future coronary disease and following an oral fat tolerance test, a short term rise in FVIIc has been observed (Lindman *et al.*, 2003), a rise which is positively associated with elevated concentrations of VLDL TG and chylomicron TG (Miller, 1998). Tissue factor is a small glycoprotein implemented in the formation of both thrombi and fibrin (Motton *et al.*, 2005) and following a meal with just a

moderate fat content, levels of circulating tissue factor appear to increase, suggesting that a typical diet, even with a moderate fat content, could promote a prothrombotic state (Motton *et al.*, 2005).

1.5 Investigating postprandial metabolism

1.5.1 Oral fat tolerance tests

An understanding of postprandial metabolism relies upon the observation of metabolic changes during the hours following a meal. The oral fat tolerance test is a valuable tool for measuring numerous metabolic responses including postprandial lipaemia (Kokalas *et al.*, 2005;Tsetsonis, 1996), glycaemia and insulinaemia (Kokalas *et al.*, 2005), endothelial function (Vogel *et al.*, 1997) and energy substrate oxidation (Stubbs *et al.*, 1995). However, to ensure the reliability and validity of data requires that pre-test conditions are standardised. Exercise and alcohol consumption can both alter postprandial metabolism, the effects of which can easily be overcome by instructing subjects to avoid alcohol and exercise for at least two days prior to the fat tolerance test. Dietary control is also important, especially if postprandial metabolism is to be monitored on more than one occasion, in which case, the same foods should be consumed for the three days preceding each trial (Gill *et al.*, 2005). In young women, there is a need to control for the menstrual cycle. Postprandial lipaemia is attenuated during the luteal phase compared to the follicular phase of menstruation. The initial glucose and insulin responses to a fat tolerance test are also different between these two menstrual phases and therefore it has been suggested that, where possible, researchers make every effort to investigate postprandial metabolism during the same phase of a woman's cycle (Gill *et al.*, 2005).

Oral fat tolerance tests may differ in the duration over which postprandial metabolism is measured with observations lasting from six to 10 hours (Gill & Hardman, 2000;Gill *et al.*, 2004;Silva *et al.*, 2005) and it is these prolonged observations that provide invaluable insight into how the body responds during the postprandial state. By graphically plotting the plasma TG concentrations during the postprandial period, the area under the response *vs.* time curve (AUC), with or without a correction for baseline values (Gill & Hardman, 2000;Gill *et al.*, 2004), can be calculated to give a summary measure of the lipaemic response. The same principle can also be used to determine other metabolic responses. A fat tolerance test may also vary in its macronutrient composition and the form that the meal takes. A high fat test meal will maximise the lipaemic response and subsequently accentuate the effect on postprandial lipaemia of any intervention under investigation. High fat tolerance tests have been provided as TG rich emulsion drinks, although these are

not always well tolerated by subjects (Whitley *et al.*, 1997) or alternatively, Cohen et al. (Cohen *et al.*, 1989) used a milkshake drink comprising cream and chocolate flavouring. More typical fat tolerance tests are presented as a meal rather than a drink, for example Whitley et al. (Whitley *et al.*, 1997) provided a test meal representative of a typical cereal product which included oats, nuts, raisins, banana, cream and milk, the fat content of which was manipulated by the addition of sunflower oil. Although an oral fat tolerance test can provide 100% fat (Whitley *et al.*, 1997) it is more common for a high fat meal to provide between 60-74% of the energy as fat (Stubbs *et al.*, 1995;Whitley *et al.*, 1997).

The relevance of the postprandial responses to such high fat meals is questionable considering that meals eaten by people in the UK rarely contain such a high fat load. Therefore it is perhaps more appropriate to observe metabolic responses to meals of mixed compositions, ones that are a better reflection of everyday dietary intakes. Kokalas et al. (Kokalas *et al.*, 2005) provided a test meal of cereal, milk, bread, ham, nuts and orange juice providing 35% of energy as fat, 50% carbohydrate and 15% protein. It is from studies such as this one using mixed meals that it became evident that postprandial responses might be influenced by the presence of other macronutrients within the meal, for example, postprandial lipaemia is augmented when carbohydrate is added to a test meal (Parks, 2001). It is interactions such as these and their subsequent effect on metabolism that are important when considering the metabolic changes that can occur in the postprandial state.

The frequent use of a single test meal protocol also warrants discussion. It is common for researchers to use a single meal, usually provided for breakfast, to investigate subsequent changes in postprandial metabolism. A typical lipaemic response to a fat tolerance test will show TG concentrations to gradually rise, peaking after three to four hours before gradually returning to baseline values within about eight hours (Frayn, 2003;Gill *et al.*, 2004). It is unlikely however that an individual will leave eight hours between meal times, and rather will initiate a second meal just four to five hours after the first. The effects of this second meal on postprandial metabolism are important. At four or five hours after a meal, plasma TG concentrations remain elevated above baseline. The arrival of a second meal then acts to further augment postprandial lipaemia so that rather than a steady return to baseline values, plasma TG concentrations will continue to rise (Silva *et al.*, 2005). The arrival of a third meal, dinner, will have the same effect, so that at the end of a normal day plasma TG concentrations will be significantly elevated above baseline. This important metabolic response during the postprandial state is overlooked when using a single test

meal study design. Although some groups have implemented a multiple test meal design (Miyashita *et al.*, 2006;Silva *et al.*, 2005), the use of such an approach remains limited.

1.5.2 Indirect calorimetry

Indirect calorimetry is a method for estimating metabolic rate and providing information on the type and rate of fuel oxidation (Frayn, 1983). Measurement of oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$) and nitrogen excretion and an understanding of the stoichiometry of carbohydrate, fat and protein oxidation provide the basic principles for this technique. The respiratory exchange ratio (RER) uses the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$ as a marker of substrate oxidation and is typically between 0.7 and 1.0; 0.7 reflecting total fat oxidation and 1.0 total carbohydrate oxidation (Ferrannini, 1988). A common technique used in indirect calorimetry requires the subject to breathe into a large, air-tight bag, the oxygen and carbon dioxide content of which is used to calculate $\dot{V}O_2$ and $\dot{V}CO_2$. A large, clear canopy or hood, placed over the subject's head, which continually extracts expired air to calculate $\dot{V}O_2$ and $\dot{V}CO_2$ is another popular method and finally, as technology has advanced, whole room calorimeters have been designed in which subjects can stay for a number of days. The non-invasive nature of indirect calorimetry makes it appealing for use within research, however, there are a number of assumptions associated with the principles of indirect calorimetry that are important to consider.

It is assumed that all oxygen consumed is used to oxidise a fuel and that all carbon dioxide produced is recovered. This may be of lesser concern for oxygen as there is no oxygen reserve within the body, however it is important when considering the large body pool of bicarbonate into which carbon dioxide produced at a cellular level may enter, rather than being excreted within expired air (Ferrannini, 1988). In situations where lactate production exceeds utilisation *e.g.* during intense exercise, an increase in hydrogen ions displaces carbon dioxide from the bicarbonate pool thus increasing carbon dioxide excretion within expired air (Frayn, 1983). Therefore, during high intensity exercise when lactate production is high, the use of indirect calorimetry to estimate fuel utilisation may be limited. Hyperventilation can occur during high intensity exercise and is another situation in which indirect calorimetry may be invalid. Hyperventilation causes carbon dioxide to be excreted at a rate exceeding oxygen consumption possibly producing RER values above 1.0 (Jequier *et al.*, 1987) which could be interpreted as pure carbohydrate oxidation rather than simply a reflection of hyperventilation. A compensatory period of hypoventilation will follow hyperventilation, during which time bicarbonate pools are replenished and if

measurements continue during both hyperventilation and hypoventilation, the calculation of substrate oxidation will generally be correct (Jequier *et al.*, 1987).

Equations used to determine energy substrate oxidation and metabolic rate are derived from single values of $\dot{V}O_2$ and $\dot{V}CO_2$ associated with the oxidation of one gram of carbohydrate, fat or protein (Jequier *et al.*, 1987). These values will however vary according to the fuel source being oxidised for example the $\dot{V}O_2$ associated with carbohydrate oxidation differs depending on whether the sugar being oxidised is mono-, di-, or polysaccharide. A $\dot{V}O_2$ of 0.746 litres is associated with glucose oxidation but increases to 0.829 litres if glycogen is preferentially oxidised (Jequier *et al.*, 1987). Using equations based on glycogen as a fuel source in a situation where glucose oxidation predominates may lead to a substantial underestimation of carbohydrate oxidation. Conversely, assuming glucose oxidation in a state where glycogen oxidation predominates will lead to an overestimation of carbohydrate oxidation (Jequier *et al.*, 1987). The same consideration should be made regarding the type and source of fat and protein being oxidised and it is important therefore that different substrate sources are considered when using indirect calorimetry. An individual's own knowledge of the physiological situation should be used to determine the appropriate fuel source (Ferrannini, 1988).

Correcting substrate oxidation and metabolic rate for protein oxidation is a topic of debate. The belief that under normal physiological conditions, protein oxidation is relatively low has led some researchers to assume zero protein oxidation (Gill *et al.*, 2004;Tsetsonis *et al.*, 1997;Wallis *et al.*, 2007). Indeed, if protein oxidation was to be miscalculated by 50%, an error of only 1.2% would occur when calculating metabolic rate. However, the same 50% would induce an error of 13% and 15% in the calculated rates of carbohydrate and fat oxidation (Ferrannini, 1988) and inclusion of protein oxidation is therefore important for the accurate determination of fuel oxidation. Protein oxidation is estimated by measuring nitrogen excretion; 1 g of nitrogen is produced for 6.25 g of protein oxidised (Abbott *et al.*, 1988;Raben *et al.*, 2003) and measuring total urinary nitrogen (TUN) and urinary urea nitrogen (UUN) are both methods for doing so. It is suggested that using UUN underestimates TUN, even with the use of a correction factor, thus measuring TUN is the recommended method (Skogerboe *et al.*, 1990). Collecting urine samples is however not always feasible and in this situation a constant rate of nitrogen excretion can be assumed for example Flatt *et al.* (Flatt *et al.*, 1985) and Melanson *et al.* (Melanson *et al.*, 2005) assumed a rate of nitrogen excretion of 0.11 $mg.kg^{-1}.min^{-1}$. An obvious limitation here is that protein oxidation may not always be constant, although it does appear relatively stable

following aerobic and resistance type exercise, periods of energy restriction (Melanson *et al.*, 2002;Schneiter *et al.*, 1995;Votruba *et al.*, 2002) and it also shows little variation following a meal rich in protein (Labayen *et al.*, 2004). Therefore, in the absence of a direct measure of urinary nitrogen, the assumption of a fixed rate of nitrogen excretion may be a valid and alternative method in some situations.

A final consideration is that indirect calorimetry assumes intermediate metabolic pathways and processes have no effect on the overall outcome, however, in situations where metabolic end products other than carbon dioxide and water are produced, such an assumption might not hold true (Frayn, 1983). Gluconeogenesis, lipolysis and ketone body production or utilisation are intermediate pathways which may alter $\dot{V}O_2$ or $\dot{V}CO_2$ and subsequently invalidate estimations of substrate oxidation. Lipogenesis and the utilisation of ketone bodies might increase the RER to a value above 1.0 whereas gluconeogenesis and ketogenesis may alter $\dot{V}O_2$ and $\dot{V}CO_2$ sufficiently enough to lower the RER below 0.7 (Frayn, 1983). Although the impact of such processes may be minimal in a normal population, the effect they can have when estimating substrate oxidation in an atypical population should be considered.

1.6 Early evidence for physical activity preventing cardiovascular disease

A number of key epidemiological studies 'paved' the way for current research investigating the association between exercise and cardiovascular health. In London, Jeremy Morris compared men who worked as bus drivers or bus conductors (Morris *et al.*, 1953), the latter considered a more active occupation, and revealed the incidence of heart disease to be significantly higher and to occur at a younger age in bus drivers than conductors. A comparison of active postal workers and sedentary civil servants similarly revealed the incidence of coronary heart disease to be lower amongst the more active postal workers (Morris *et al.*, 1953). In America, Ralph Paffenbarger studied the activities of San Francisco longshore men, categorising them according to their workload of light, moderate or heavy loads. A heavy workload provided the greatest protection against mortality from heart disease in workers of all ages, with greatest benefit in older men. Those engaged in light and moderate work suffered almost twice the risk of fatal heart disease than those in heavy work (Paffenbarger & Hale, 1975).

These findings were extended to investigate the benefit of leisure time physical activity for cardiovascular health. Morris (Morris *et al.*, 1980) recruited around 18,000 male government workers collecting information on the amount of vigorous sports and

recreations or heavy work (digging, gardening) they participated in. Vigorous exercise was associated with a 40% and 50% lower risk of suffering fatal and non-fatal heart attacks, respectively, than no vigorous exercise, findings which were extended to the sub-groups who were smoking or non-smoking, obese or lean, in good health or poor health and with or without family history of coronary disease. In a series of studies, Paffenbarger investigated the impact of leisure time physical activity in men previously students at Harvard University, the Harvard Alumni Study. They revealed that 'light sports' were no more protective than doing no sport at all and a sedentary lifestyle was associated with a 38% higher risk of heart attack (Paffenbarger, Jr. *et al.*, 1978). Vigorous activity levels were associated with lower mortality and a greater energy expenditure (above 2000 kcal per week) was associated with a reduction in heart disease mortality. Paffenbarger also introduced the need to remain active throughout life (Paffenbarger, Jr. *et al.*, 1978;Paffenbarger, Jr. *et al.*, 1984), revealing that men who were highly active at university but did not continue vigorous exercise had no lower risk for coronary disease than men who were inactive throughout.

In addition to the work of Morris and Paffenbarger research has continued to investigate the effect of physical activity on cardiovascular health, work which has extended findings to show low levels of physical activity are also associated with adverse risk profiles for CVD in developing countries (Forrest *et al.*, 2001). A large population based study from Finland (Barengo *et al.*, 2004) reported both moderate and high levels of physical activity lowered total mortality and cardiovascular risk in men and women aged 30 to 59 years. In the same study cohort, moderate and high levels of occupational activity had a similar effect lowering total mortality and cardiovascular risk by up to 27% compared to low activity levels. A meta analysis from Lee *et al.* (Lee *et al.*, 2003) summarising the findings from 23 studies also concluded that stroke incidence was lowered by 27% in highly active individuals compared to non-active. A similar effect also evident in those who were moderately active compared to inactive. A dose-response effect of physical activity has been described and Lee and Skerret (Lee & Skerrett, 2001) revealed a weekly energy expenditure of approximately 1000 kcal to be associated with lower mortality, with moderate or high intensity exercise inducing energy expenditures above such a threshold having no greater effect on lowering risk.

1.7 Exercise training and postprandial metabolism

Cross sectional studies have revealed metabolic differences between trained and untrained, active and inactive populations. Oral fat tolerance tests completed by 29 male athletes and

29 matched control subjects revealed postprandial lipaemia to be significantly attenuated in athletes compared to controls (Cohen *et al.*, 1989). Similarly Merril *et al.* (Merrill *et al.*, 1989) reported the postprandial lipaemic response to a fat meal to be lower in young trained men compared to their untrained sedentary peers. The insulin response to an oral glucose load is also lower in trained than untrained men, suggesting a role for regular exercise in enhancing insulin sensitivity (King *et al.*, 1987).

Longitudinal studies where cohorts' complete prolonged training programmes have also been implemented. In 1983 Patsch and Colleagues (Patsch *et al.*, 1983) followed a single subject over three years as he progressed from being sedentary to running approximately 40-45 miles per week. On completion of the training period, postprandial lipaemia was attenuated by 95% compared to pre-training. Weintraub *et al.* (Weintraub *et al.*, 1989) assigned six normolipidemic men to a running intervention of approximately 15 miles per week and the response to an oral fat tolerance test showed chylomicron TG to be 37% lower and non-chylomicron TG to be 28% lower, although the latter effect just failed to reach significance. Some years later in 1992, Drexel *et al*. (Drexel *et al.*, 1992) implemented a 12 week aerobic exercise programme in 11 healthy, overweight women, reporting postprandial TG to be significantly attenuated post training, although an associated weight loss of 4.3 kg makes it difficult to ascertain whether it was exercise or the lower body mass that mediated these responses. In 97 sedentary men, a nine month exercise programme significantly lowered both glucose and insulin responses to an OGTT indicating enhanced insulin sensitivity (Dengel *et al.*, 1998) and a similar response has been observed after a shorter exercise programme of 16 weeks (Cox *et al.*, 2004). Improvements in vascular function have also been observed; a relatively high intensity eight week cycling programme induced a 5% reduction in arterial pulse wave velocity (PWV) (Kakiyama *et al.*, 2005) and a 16 week walking and jogging programme also lowered aortic PWV (Hayashi *et al.*, 2005).

Although the metabolic benefits of training are consistent they may not be a true training effect but rather an acute response to the final exercise session. In training studies, it is uncommon for researchers to instruct subjects to stop exercising for any length of time prior to a fat tolerance test and because regular exercise becomes integrated into daily routines, it is likely that unless specifically asked not to, subjects exercise during the days immediately preceding metabolic testing (Malkova & Gill, 2006). This makes it difficult to distinguish whether changes in postprandial metabolism are a training adaptation or simply an acute response to prior exercise. In support of an acute effect, Aldred *et al.*

(Aldred *et al.*, 1995) showed that in sedentary women, the TG response to a fat tolerance test determined two days after a twelve week walking intervention had ceased, was not different compared to rest despite significant increases in aerobic fitness and a reduction in body fat.

Detraining studies also provide insight into the nature of exercise-induced metabolic changes. Herd and colleagues (Herd *et al.*, 1998) assigned young recreationally active adults to a 13 week running programme or control period and fat tolerance tests were completed prior to intervention and 15 hours, 60 hours and nine days after the final exercise session. At 60 hours no significant difference in the postprandial TG, glucose or insulin response was observed compared to pre-exercise values and when compared to TG measured at 15 hours, the postprandial TG response was 37% and 46% higher at 60 hours and 9 days, respectively, suggesting a rapid increase in lipaemia as detraining progressed. Hardman and colleagues (Hardman *et al.*, 1998) recruited 10 endurance athletes who ceased all training for one week, during which fat tolerance tests were completed at 15 hours, 60 hours and 6.5 days post-exercise. Similar to Herd *et al*. the authors reported significant increases in postprandial TG concentrations at 60 hours and 6.5 days, a majority of the increase occurring within the initial 60 hours. The postprandial insulin response was also higher after 6.5 days of de-training (Hardman *et al.*, 1998). More recently, Gill *et al.* (Gill *et al.*, 2003b) showed a week of detraining to significantly elevate postprandial lipaemia (53%) an effect which was predominantly accounted for by an increase in chylomicron TG rather than VLDL. In the same study, postprandial insulinaemia increased by 38% in the absence of exercise and interestingly an increase in TNF-α with detraining suggested that exercise reduced inflammation and detraining promoted a proinflammatory state. Evidence suggests that in the absence of recent exercise, any beneficial effect of exercise on metabolism, at least with regard to postprandial lipaemia, insulinaemia and perhaps inflammation, is lost. It is likely therefore that these exercise-induced metabolic changes are a response to regular and repeated episodes of exercise rather than prolonged aerobic training, although the chronic physiological adaptations associated with such training are likely to augment this acute exercise effect.

1.8 Acute exercise and postprandial metabolism

To investigate the acute effect of exercise on postprandial metabolism, studies have implemented experimental protocols where the postprandial metabolic responses to a single session of exercise are measured after a delay of some hours, usually overnight, or during the immediate post-exercise period. An overview of these protocols is given in

Figure 1.1. It should be noted however, that these are basic protocols which will be individually tailored to suit the specific needs of a research group.

1.8.1 Acute exercise and postprandial lipaemia

One of the earliest studies to investigate the effects of acute exercise on postprandial lipaemia was published in 1962 and investigated the effects of a 16 km march performed after an oral fat tolerance test in 20 healthy army recruits compared to 20 recruits who were bed bound (Nikkila & Konttinen, 1962), and on completion of the march, TG concentrations were 39% lower compared to those in bed. This beneficial effect of exercise performed in the postprandial state has since been confirmed by others (Hardman & Aldred, 1995;Zhang *et al.*, 1998).

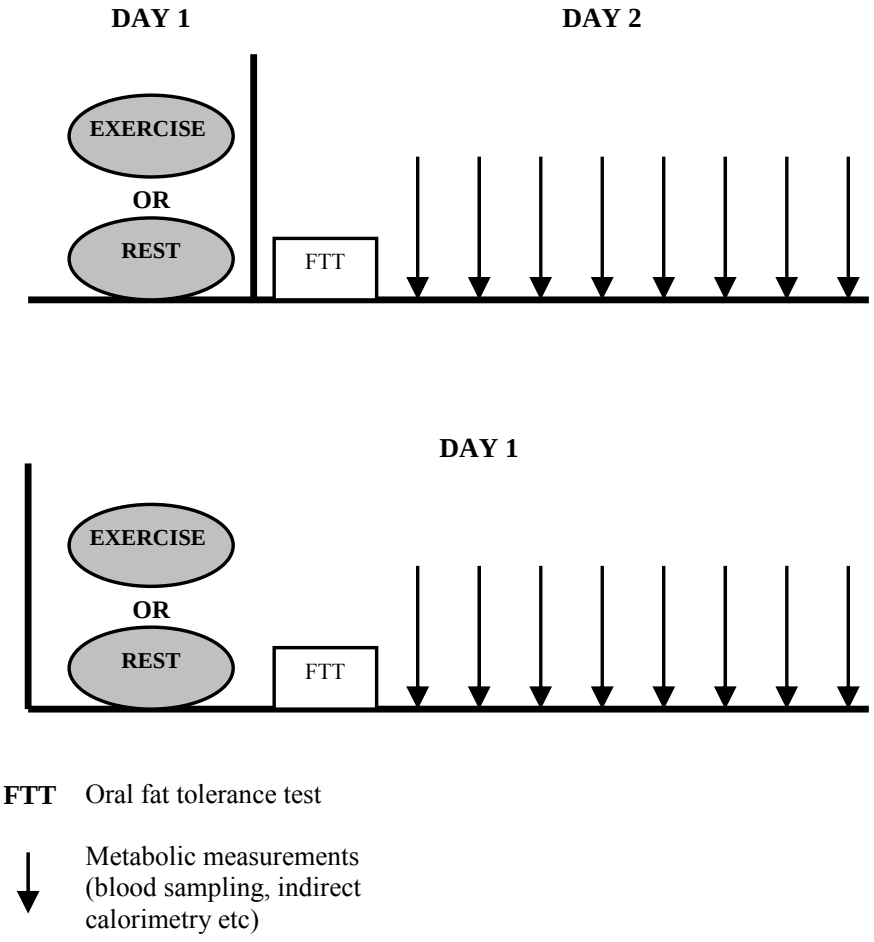


Figure 1.1 An overview of the two-day (top) and one-day (bottom) exercise protocols.

It is more recently that the effects of prior exercise on postprandial metabolism have been addressed. One of the earliest studies from Aldred *et al.* (Aldred *et al.*, 1994) had 12 subjects complete two separate two-day trials with either a two-hour moderate intensity treadmill walk or a rest period performed the afternoon prior to a fat tolerance test. The

authors reported the postprandial lipaemic response to be approximately 31% lower following exercise compared to rest. In the years following this study, the effect of acute exercise on postprandial lipaemia has been extensively investigated.

In middle-aged, untrained men a 90-minute treadmill walk at 60% $\dot{V}O_2$ max attenuated postprandial TG concentrations by 25% (Gill *et al.*, 2001a) and in untrained women, 90 minutes of treadmill walking reduced the postprandial TG response by approximately 16% (Tsetsonis *et al.*, 1997). The effect of acute exercise on postprandial lipaemia in trained subjects appears augmented with trained men and women having 51% and 30% lower TG concentrations, respectively (Tsetsonis *et al.*, 1997;Zhang *et al.*, 1998). An acute exercise effect is also observed in pre-menopausal and post-menopausal women; two hours of moderate intensity walking attenuated postprandial TG by 18% in pre-menopausal women (Gill *et al.*, 2003a) and a similar reduction of 20% was observed in post-menopausal women the day following 90 minutes of moderate intensity walking (Gill & Hardman, 2000). Encouragingly, not just adults seem to benefit and both continuous and intermittent games activities have been shown to attenuate postprandial TG responses in young adolescent males (Barrett *et al.*, 2007). An acute effect of exercise has also been investigated in populations at increased CVD risk and a 25% lowering of postprandial TG in centrally obese men has been reported (Gill *et al.*, 2004). A recent study did however fail to report any effect of a 90 minute treadmill walk performed the day before an oral fat tolerance test on postprandial lipaemia in 10 men with type 2 diabetes (Gill *et al.*, 2006a), data contrasting with those above and highlighting the need for future research to investigate the effect of acute exercise in non-healthy populations.

Zhang *et al.* (Zhang *et al.*, 1998) investigated the effect of exercise timing on postprandial TG concentrations and showed exercise performed one hour prior to a fat tolerance test to lower TG by 38% whereas exercise 12 hours prior to a fat tolerance test lowered postprandial TG by 51%. Ferguson *et al.* (Ferguson *et al.*, 1998) reported no change in fasting TG immediately after exercise of differing intensities but when measured again 24 hours later, exercise significantly attenuated TG. Therefore it appears that the TG lowering effect of acute exercise is not immediate and is maximised after a delay of some hours. The energy expended during an exercise session also seems important for determining the magnitude of lower TG. Twelve subjects walked for 90 minutes at either 30% or 60% $\dot{V}O_2$ max the afternoon prior to an oral fat tolerance test and postprandial lipaemia was 26% and 16% lower, following the moderate and low intensity exercise sessions, respectively (Tsetsonis & Hardman, 1996). In addition, for women walking at

50% $\dot{V}O_2$ max for one or two hours, the effect on postprandial lipaemia was more than doubled with 9% and 23% lower TG concentrations, respectively (Gill *et al.*, 2002a). Similarly, when nine volunteers walked for three hours at 30% $\dot{V}O_2$ max or for 1.5 hours at 60% $\dot{V}O_2$ max, both inducing the same energy expenditure, the reduction in postprandial lipaemia measured the following day was 33% for both (Tsetsonis, 1996).

1.8.2 Acute exercise, postprandial insulinaemia, glycaemia and insulin resistance

Plasma glucose concentrations seem fairly resistant to change both immediately and the day following acute exercise. Measured immediately post-exercise glucose remains unchanged whether exercise is performed before or after a fat tolerance test (Katsanos & Moffatt, 2004) or following a high intensity cycling intervention (Venables *et al.*, 2007), although a post-exercise increase in glucose has recently been reported (Broom *et al.*, 2007). In men with type 2 diabetes, two studies have reported moderate and high intensity exercise performed after a test breakfast to lower the immediate glucose response, however such an effect appeared to be transient (Larsen *et al.*, 1997;Larsen *et al.*, 1999) and as such may explain the absence of any obvious change in glucose measured the day following exercise. In men with type 2 diabetes, 90 minutes of exercise failed to attenuate postprandial glucose concentrations (Gill *et al.*, 2006a) findings similar to earlier data showing no lowering of glucose the day following exercise in healthy men and women (Tsetsonis & Hardman, 1996). Similar to the immediate response, one study has also reported a slight increase in postprandial glucose concentrations (Gill *et al.*, 2002b) the day following exercise.

With regard to insulin, the day following exercise postprandial insulin responses were attenuated by 11% in men with type 2 diabetes (Gill *et al.*, 2006a) and a similar response was observed in centrally obese men (Gill *et al.*, 2004). In healthy normal weight men and women the postprandial insulin response was attenuated by exercise, whilst a significant reduction in HOMA estimated insulin resistance was observed in men only (Gill *et al.*, 2002b). Immediately post-exercise, Englert et al. (Englert *et al.*, 2006) reported postprandial insulin concentrations to be 28% lower compared to no exercise and immediately following one hour of cycling, Venables et al. (Venables *et al.*, 2007) reported a 10% lowering in postprandial insulin concentrations compared to control, although this did not reach significance. Conversely, in their study, Katsanos and colleagues (Katsanos & Moffatt, 2004) failed to uncover any immediate effect of exercise performed before or after a test meal on postprandial insulinaemia. In addition to insulin concentrations *per se*, a single session of exercise also appears useful for enhancing insulin sensitivity. In seven

healthy, untrained young men who completed three euglycemic hyperinsulinaemic clamps after a period of rest, immediately after 60 minutes of cycling and 48 hours after the same exercise intervention, Mikines *et al.* (Mikines *et al.*, 1988) reported both sensitivity and responsiveness to insulin to be significantly increased immediately after exercise and when measured again at 48 hours.

A concomitant reduction in both postprandial TG and insulin after exercise, and a significant association between the two variables has led to the suggestion that changes in TG may be related to changes in insulin or vice versa (Thompson *et al.*, 2001). Gill *et al.* did indeed report a correlation between insulin sensitivity and TG when in the unexercised state but when they extended their investigation into the exercised state and investigated the exercise-induced changes in TG and insulin, the relationship was no longer evident (Gill *et al.*, 2002b). Furthermore, in men with type 2 diabetes, a significant decline in postprandial insulin concentrations has been observed in the absence of any change in the TG response (Gill *et al.*, 2006a) and in healthy lean men, a reduction in postprandial TG was observed without any change in insulin (Gill *et al.*, 2004). Therefore it seems that, in the short-term at least, exercise-induced changes in postprandial TG and insulin responses are mediated by two different mechanisms.

1.8.3 Acute exercise and energy substrate utilisation

The extent to which exercise induces greater fat oxidation depends on the intensity and duration of exercise and the fitness level of the exerciser themselves (Hansen *et al.*, 2005). Moderate intensity exercise promotes fat oxidation, whilst at higher intensities the body relies upon carbohydrate: Two separate studies have identified 65% $\dot{V}O_2$ peak and 64% $\dot{V}O_2$ peak as the work rates at which the rate of fat oxidation is maximal (Achten *et al.*, 2002;Romijn *et al.*, 1993). The rate of energy substrate utilisation during exercise may also be influenced by the macronutrient composition of the pre-exercise diet, particularly the fat composition, and the timing of the pre-exercise diet in relation to the exercise session itself (Hansen *et al.*, 2005).

In 2002, Votruba *et al*. (Votruba *et al.*, 2002) published the first of three studies investigating the effects of acute exercise on postprandial fat utilisation. Seven young women completed two hours of rest, two hours of light intensity cycling (25% $\dot{V}O_2$ peak) and a shorter period of heavy load cycling (85% $\dot{V}O_2$ peak) all performed within a respiratory chamber. During the postprandial period, fat oxidation was significantly

elevated, by approximately 12 g, following both the light and heavy workloads compared to rest. Carbohydrate utilisation was significantly elevated in the heavy exercise trial compared to both the rest and light exercise trials (Votruba *et al.*, 2002). In two subsequent studies (Votruba *et al.*, 2003;Votruba *et al.*, 2005), Votruba and colleagues reported that light and moderate intensity exercise stimulated an increase in fat utilisation and extended their earlier findings to show that this increase was accounted for by a greater oxidation of dietary monounsaturated fat rather than saturated fat. Interestingly, even when a high carbohydrate load is consumed after exercise, fat oxidation remains elevated suggesting that post-exercise, the body favours lipids as a fuel source over carbohydrate (Kimber *et al.*, 2003;Melby *et al.*, 2002).

Beyond the immediate effect of exercise, fat oxidation remains elevated for a number of hours. Kiens and Richter reported the RER to be significantly lower following 90 minutes of exhaustive exercise when measured 18 hours post-exercise (Kiens & Richter, 1998). Tsetsonis et al. (Tsetsonis *et al.*, 1997) determined postprandial fat oxidation in trained and untrained women the day following 90 minutes of walking at 60% $\dot{V}O_2$ max or an equivalent period of rest and they showed increases in postprandial fat oxidation of 28% in the untrained and 41% in the trained subjects. Two further studies published in 2001 also support a role for exercise in mediating postprandial substrate oxidation. Ninety minutes of cycling by eight young men significantly lowered the postprandial respiratory exchange ratio and attenuated postprandial carbohydrate oxidation by 24%, when measured the following day (Herd *et al.*, 2001), with a 30% increase in postprandial fat oxidation failing to reach significance. In eleven healthy men, Gill *et al*. (Gill *et al.*, 2001a) reported 90 minutes of walking to lower total postprandial carbohydrate oxidation and increase total fat oxidation, an effect which appeared to be mediated by increases in both endogenous and exogenous fat oxidation.

1.8.4 Acute exercise, endothelial function and PWV

Only recently has a beneficial effect of acute exercise on postprandial endothelial function been reported. In 2004, Gill *et al*. (Gill *et al.*, 2004) measured fasting and postprandial endothelium-dependent and endothelium-independent vasodilatory responses the day following a moderate intensity 90-minute treadmill walk and reported that exercise increased fasting and postprandial endothelium-dependant vasodilation by 25% and 15%, respectively. Surprisingly, there have been few additional studies investigating the effects of prior exercise on postprandial endothelial function. A study in 2006 measured the effect of walking two hours after a fat meal on postprandial flow mediated dilation in eight

healthy adults (Padilla *et al.*, 2006) and reported flow mediated dilation to be significantly lower four hours after the meal when compared to dilation following a meal with no exercise.

A number of studies are, however, available investigating the acute affect of exercise on PWV although a majority of these have not used an oral fat tolerance test. Fifty sedentary adults completed a maximal treadmill test, whilst in a fasted state, following which both upper and lower limb PWV were recorded for a 60-minute recovery period (Naka *et al.*, 2003). A rapid incline in upper limb PWV was quickly followed by a gradual decline over the 60 minutes and lower limb PWV rapidly declined initially before steadily increasing. At 60 minutes of recovery however, both upper and lower limb PWV were approximately 10% lower than baseline values. One study which has studied the postprandial state measured central and peripheral PWV in 12 sedentary men following 30 minutes of cycling performed after a light breakfast (Kingwell *et al.*, 1997b). Central and femoral PWV were reduced by 5% and 10%, respectively, when measured 30 minutes post-exercise but when measured again at 60 minutes, both had returned to pre-exercise values. Heffernan *et al*. (Heffernan *et al.*, 2007a) have also very recently reported exercise-induced changes in PWV in 13 young, active men where 20 minutes after a 30 minute cycling intervention postprandial central and peripheral PWV were attenuated by 9% and 7%, respectively. In the same year, Heffernan and colleagues (Heffernan *et al.*, 2007b) also reported peripheral PWV to be lower in resistance trained men and healthy controls 30 minutes after a maximal treadmill test, although no change in central PWV was observed. To the author's knowledge, only one study has investigated the effect of prior exercise on postprandial PWV. Clegg *et al.* (Clegg *et al.*, 2007) recruited eight young, recreationally active men who completed an oral fat tolerance test preceded by 60 minutes of moderate intensity exercise or rest. Here, peripheral PWV was lower following exercise compared to rest when measured two and four hours after the meal and exercise also successfully diminished the postprandial rise in PWV that was observed in the rest trial.

It does appear that acute exercise lowers postprandial PWV measured during the short-term post-exercise recovery period. It remains unclear however as to how long such an effect persists as none of the above studies have measured exercise-induced changes in PWV beyond 4 hours. Furthermore, all of the subject cohorts have been healthy, normal weight and predominantly male, thus whether such a response of PWV to exercise will be observed in diseased, overweight and female populations remains to be investigated.

1.9 Potential mechanisms mediating exercise-induced changes in postprandial metabolism

An exercise-induced lowering in postprandial TG concentrations might be explained by a reduction in the appearance or an increased clearance of TG and possibly changes in chylomicron and VLDL particle structure. A reduced appearance of chylomicrons into plasma is suggested an unlikely mediator of lower TG concentrations (Malkova & Gill, 2006) but the enhanced clearance of chylomicron particles from the circulation may be important. Postheparin lipoprotein lipase activity, a measure of whole body lipoprotein lipase activity, is increased following exercise training and prolonged periods of strenuous exercise. Five consecutive days of cycling training increased lipoprotein lipase mRNA by 127% four hours post-exercise and increased lipoprotein lipase mass by 93% eight hours post-exercise (Seip *et al.*, 1997). Furthermore, lipoprotein lipase activity measured in eight trained men six and 18 hours after 90 minutes of exhaustive exercise was 37% and 72% higher, respectively (Kiens & Richter, 1998). Although increased lipoprotein lipase activity is likely to be important, its role in mediating the lower TG response to acute exercise is less convincing. Following acute moderate intensity exercise, the increase in lipoprotein lipase activity is somewhat smaller compared to strenuous exercise (Ferguson *et al.*, 1998;Zhang *et al.*, 2002). Furthermore, a simultaneous increase in lipoprotein lipase activity and reduction in plasma TG is not always observed. Gill *et al*. (Gill *et al.*, 2003a) reported prior exercise to lower postprandial TG concentrations by 18%, although no significant change in lipoprotein lipase activity was observed. Similarly, 24 hours after 60 minutes of moderate exercise, TG concentrations were attenuated by 10% but LPL activity did not differ (Ferguson *et al.*, 1998). Finally, one study has shown that a lipid emulsion, designed to mimic the appearance of chylomicron particles, is not cleared any faster the day following acute exercise, despite a significant reduction in plasma TG concentrations (Gill *et al.*, 2001b). Thus the evidence suggests that altered chylomicron metabolism may not be the key mediator of lower TG concentrations.

The evidence for an exercise effect on VLDL metabolism is more encouraging. In response to 90 minutes of walking, postprandial chylomicron and VLDL particles were reduced by 29% and 57%, respectively (Gill *et al.*, 2006b). Fewer VLDL particles may be a consequence of attenuated hepatic VLDL secretion or greater VLDL clearance, although currently, it is not clear which of these pathways is more dominant. Indirect evidence for a reduction in the hepatic secretion of VLDL particles is provided by measuring 3-hydroxybutyrate (3-OHB) concentrations, an increase in which would indicate greater hepatic fat oxidation (Williamson & Whitelaw, 1978) and lower VLDL secretion. The day

following 90 minutes of walking, a 60% increase in postprandial 3-OHB concentrations has been observed (Gill *et al.*, 2001a) with a rise of 32% also evident in men with type 2 diabetes (Gill *et al.*, 2006a). Significant correlations between exercise-induced changes in fasting 3-OHB and exercise-induced changes in fasting and postprandial TG concentrations further suggest a reduction in VLDL secretion might contribute to lower TG concentrations (Gill *et al.*, 2006a). In the post-exercise state it therefore appears that within the liver, fatty acids are directed towards oxidation rather than secretion within VLDL into the plasma (Malkova & Gill, 2006). VLDL clearance may also be enhanced post-exercise (Gill *et al.*, 2006b); the day following exercise the apo CII/apo B ratio was increased whilst both the apo CIII/apo B and apo E/apo B ratios were decreased, structural changes facilitating the clearance of VLDL from plasma (Gill *et al.*, 2006b).

Both immediately (Marion-Latard *et al.*, 2003;Mougios *et al.*, 2003) and the day following exercise (Gill & Hardman, 2000;Gill *et al.*, 2004) increases in plasma NEFA concentrations might mediate an increase in whole body fat oxidation; in competition with glucose, fatty acids are the preferred substrate for oxidation (Randle *et al.*, 1963). Increases in hepatic fat oxidation will contribute to greater whole body fat oxidation but it is likely that exercise-induced changes in fat oxidation in other tissues are also important. An increase in the oxidation of skeletal muscle TG stores or intramuscular TG (IMTG) has been reported with Kiens and Richter showing lower IMTG concentrations three hours post-exercise reaching a maximal reduction of 20% after 18 hours of recovery (Kiens & Richter, 1998). These reductions in IMTG appear to precede a gradual replenishment of muscle glycogen occurring at 6 hours of recovery with a return to baseline by 30 hours, suggesting that the body preferentially oxidised fat to facilitate muscle glycogen recovery (Kiens & Richter, 1998). Another study has however failed to show exhaustive exercise to have any effect on IMTG stores, although they did report that their methods may have overlooked any small significant increases in IMTG utilisation, and instead suggested that circulatory lipids such as fatty acids and TG were a more important source for increased fat oxidation (Kimber *et al.*, 2003).

The effect of exercise on glycaemia may be mediated by changes in glucose transport associated with improved insulin sensitivity (Henriksen, 2002). Increased translocation of GLUT-4 transporters may enhance glucose uptake and insulin action and Kennedy et al. (Kennedy *et al.*, 1999) reported GLUT-4 protein expression to increase by 71% and 74% in healthy subjects and patients with type 2 diabetes, respectively, following 45-60 minutes of moderate to high intensity cycling. A more recent study has also shown GLUT-4

protein content in skeletal muscle to increase following low and high intensity exercise by 106% and 61%, respectively (Kraniou *et al.*, 2006). Glycogen depletion may facilitate improvements in insulin sensitivity (Wojtaszewski *et al.*, 2002); in rodents insulin sensitivity is enhanced following glycogen depleting exercise, an effect which persists when carbohydrate intake is restricted (Cartee *et al.*, 1989). Furthermore, insulin stimulated recruitment of GLUT-4 is increased with lower glycogen whilst glycogen supercompensation restricts insulin action (Derave *et al.*, 1999;Kawanaka *et al.*, 1999) and in humans, eating 100g of glucose following glycogen depleting exercise eliminates any exercise-induced increase in insulin action (Bogardus *et al.*, 1983). It is likely, therefore, that changes in glycogen might mediate insulin activity and sensitivity in the post-exercise period.

Enhanced endothelial function, lower arterial stiffness and lower PWV in the post-exercise state are a likely response to exercise-induced changes in nitric oxide, a molecule enhancing vasodilation within a blood vessel (Boger *et al.*, 2003). In trained and untrained volunteers, two hours of exercise increased plasma nitrate, a product of nitric oxide metabolism, by 18% and 16%, respectively (Jungersten *et al.*, 1997) and in 15 young men, 30 minutes of cycling increased plasma nitrate/nitrite concentrations immediately post exercise (Tozzi-Ciancarelli *et al.*, 2002). It is not possible to determine exactly how long these effects last although when measured 24 hours after exercise, no difference in the nitrate/nitrite content compared to resting values was observed (Tozzi-Ciancarelli *et al.*, 2002). Increased blood flow, which may last for up to 16 hours post-exercise (Malkova *et al.*, 2000), may also contribute to greater nitric oxide availability due to increased shear stress placed on the endothelium. Changes in TG concentrations may also facilitate enhanced vascular function following acute exercise. Impaired endothelial function and elevated PWV are associated with elevated TG concentrations (Legedz *et al.*, 2006;Vogel *et al.*, 1997) and changes in PWV have been shown to correlate with changes in postprandial TG concentrations (Daskalova *et al.*, 2005). It is possible therefore that an exercise-induced lowering of TG the day following exercise may contribute to enhanced endothelial function and arterial stiffness.

1.10 Exercise and metabolism: The unknown role of energy balance

A large volume of research is available investigating the effects of acute exercise on postprandial metabolism but it remains unclear to what extent exercise-induced changes in postprandial metabolism are mediated by the associated energy deficit or exercise *per se*. A need to clarify the role for an exercise-induced energy deficit in mediating changes in

postprandial metabolism arises from data provided by Gill and colleagues (Gill & Hardman, 2000). They compared the effects of exercise and dietary-induced energy deficits, of similar magnitudes, on postprandial TG concentrations and reported that in response to an oral fat tolerance test and compared to the control trial, there was a three fold greater reduction in postprandial TG concentrations following exercise compared to energy intake restriction. Two possible conclusions could be drawn. Firstly that an energy deficit is not important for attenuating postprandial TG concentrations or secondly, that an energy deficit is important but exercise and dietary-induced energy deficits have a different effect on postprandial metabolism. To clarify which hypothesis is true, there is a need to compare the acute effects of exercise both with and without an energy deficit on postprandial lipaemia, however, to this end and to the author's knowledge, no attempt has been made to do so.

Indirect evidence for the effect of exercise without energy deficit is provided by training studies with no associated weight loss. In sedentary women, a 16-week aerobic training programme with no change in body mass significantly lowered TG and increased HDL concentrations compared to pre-training (LeMura *et al.*, 2000). In another study, a six-month training programme failed to alter TG or cholesterol concentrations, although a significant increase in insulin sensitivity was observed (Duncan *et al.*, 2003). The absence of any change in subject $\dot{V}O_2$ max in the latter study does, however, suggest that the training intensity was not sufficient to alter lipid metabolism. One potential limitation with training studies is that although body mass does not change, fat mass might be altered, therefore contributing to changes in metabolism. Thompson *et al.* (Thompson *et al.*, 1997), however, implemented a 12-month training programme during which no change in BMI or percentage body fat was observed and they reported a 10% elevation in postprandial HDL cholesterol and a 7% decline in postprandial TG concentrations. More recently, a 12-week training programme completed by overweight and obese young girls (Nassis *et al.*, 2005), again with no change in BMI or percentage body fat, revealed insulin concentrations to be 28% lower following exercise. No change in TG was observed although this may have been a consequence of the three day delay between the final exercise session and metabolic testing (Nassis *et al.*, 2005). A limited number of studies have made a direct attempt to control for the effect of exercise-induced energy deficits during training. Eight sedentary men completed a six-week exercise programme, during which their energy intake was increased to replace the energy expended during exercise and maintain energy balance (Branth *et al.*, 2005). There were no changes in body mass or percentage body fat following the intervention but the authors also failed to report any

effect of training on blood lipid profiles. Another study published the same year had eight sedentary and overweight subjects complete six consecutive days of moderate intensity treadmill walking, each expending approximately 500 kcal (Black *et al.*, 2005). During the six days, energy expended during the walks was replaced using high carbohydrate drinks and snacks. When measured 24 hour after the final exercise session, the authors reported no change in insulin concentrations or insulin action and also failed to observe any change in TG or cholesterol concentrations.

At present, the effect of exercise training without an energy deficit is unclear and warrants further investigation. It is important to consider however that with the exception of a few (Duncan *et al.*, 2003;Nassis *et al.*, 2005;Thompson *et al.*, 1997), studies have measured the effects of exercise on fasting metabolism and the relevance of their findings to a real world situation where people spend a majority of their time in the postprandial state may be limited. Furthermore, all of these studies have used longer intervention protocols and to the author's knowledge no study has investigated the effect of a single session of moderate intensity exercise with the energy deficit replaced on fasting and postprandial metabolism. Considering the benefits of acute exercise discussed above, it is important to understand the role for an acute state of energy deficit or energy balance in mediating exercise-induced changes in postprandial metabolism.

1.11 Appetite and feeding behaviour

The term appetite is used to collectively describe different sensations that are related to and determine food intake (Sorensen *et al.*, 2003) and is suggested to include three main elements; hunger, satiety and satiation (Mattes *et al.*, 2005). Hunger is a feeling indicative of food deprivation and the need to initiate food intake (Sorensen *et al.*, 2003). Satiation determines how much food is eaten and the duration of the food intake whilst satiety is a feeling that occurs following a meal determining the inter meal duration (Mattes *et al.*, 2005;Sorensen *et al.*, 2003).

Active individuals who regularly exercise seem better able to regulate their energy intake and energy expenditure, maintaining stable body weights and preventing weight gain (Long *et al.*, 2002). This coupling between energy intake and energy expenditure appears disrupted in obese populations as they seem less able to modify their appetite, subsequently increasing food intake above that which is required. Physiological differences in the levels of circulating appetite hormones, such as leptin, in those who are overweight and obese may contribute to impaired appetite control (Considine *et al.*, 1996) but it might also be a

result of the greater pleasure and increased palatability of foods experienced by those who are overweight or alternatively due to the consumption of more energy rich foods and larger portion sizes (Rolls, 2007). Those who are obese might also be less sensitive to the satiety signals derived from food (Long *et al.*, 2002), a possible cause of which could be the more rapid consumption of food at meal times (Rolls, 2007). An ever increasing availability of relatively cheap but appealing foods will only exacerbate the current problem of obesity, and thus there is a growing need to understand why some populations are better able to match their energy intake to their daily energy demands than others. Furthermore, there is a need to understand how interventions such as exercise might be successful in modifying appetite and subsequent feeding behaviour in order to treat and manage obesity.

1.12 Measuring appetite and feeding behaviour

1.12.1 Real world vs. laboratory based studies

Studies researching appetite control use either a tightly controlled laboratory protocol or a more typical real world environment, both of which have their benefits and their limitations. By studying food intake and appetite within the laboratory, researchers can control for many variables that might influence the outcome of interest (De Castro, 2000), variables such as subject population, food cost, time of day, food composition and social interaction. This allows for accurate conclusions to be drawn with regard to the variable under investigation and its effect on appetite. However, whist such controls ensure valid data, the laboratory is a somewhat artificial setting and the magnitude of the effect that the variable might have in a real world situation is not assessed and may be over emphasised (Bellisle, 1999;De Castro, 2000). Experiments based within the real world situation of the home or a busy canteen enable appetite and feeding behaviour to be monitored within a more typical environment and the conclusions drawn from such studies might therefore prove more relevant (De Castro, 2000). One limitation with studies performed outside of the laboratory is, however, that if an effect of any intervention is uncovered it is unlikely that firm conclusions can be drawn on the exact causative factor as so many other variables, which could not be controlled for, may have also influenced the outcome. Although both types of study design have their limitations, both are necessary to help us further our understanding of how appetite is regulated and feeding behaviour controlled, and their use should perhaps be determined by the nature of the research study.

1.12.2 Direct measurement of food intake

One method for investigating appetite and feeding behaviour is to measure food intake within a laboratory or canteen environment. To measure food consumption experimenters provide subjects with a buffet meal from which they are instructed to eat ad libitum (Martins *et al.*, 2007a;Pomerleau *et al.*, 2004). Foods in the meal are typically presented in excess quantities to those that would be expected to be consumed and prior to being given to the subject, each individual component of the buffet meal is either counted or weighed. The buffet meal can be provided within the laboratory itself (Martins *et al.*, 2007a;Pomerleau *et al.*, 2004) or alternatively within a canteen or cafeteria type area (George & Morganstein, 2003) providing a more natural setting for the subject. Typically, subjects are instructed to eat until they reach their usual degree of satiation and are satisfied (Imbeault *et al.*, 1997), following which the food is re-counted or re-weighed to enable the calculation of energy and macronutrient intake, which is typically achieved using computerised food databases and manufacturers' nutrition labels.

There are some important considerations for designing and implementing a buffet style meal from which energy and macronutrient intake will be determined. The portion size effect should be considered as previously learned behaviours may influence normal food intake (Herman & Polivy, 2005). For example, over a period of time, an individual might learn that a sandwich made with two slices of bread is the accepted and normal amount to eat in a meal. Thus if a subject is presented with sandwiches that are cut into halves, or quarters, they will typically consume two half sandwiches or four quarter sandwiches; the equivalent to one whole sandwich. It may be more appropriate to provide an uncut loaf of bread, a block of cheese and a large quantity of butter as this removes any effect of portion size on food consumption. This same principle holds for a number of different foods; provide crisps in a bowl rather than individual packets or provide a whole cake rather than individual slices etc. There is also a potential problem with subjects overeating at a buffet meal because cost restrictions have been removed (De Castro, 2000) or alternatively under eating because they believe that their food intake will be measured and 'judged' in some way by the experimenters (Bock & Kanerek, 1995). It is also possible that subjects might consume less food and eat more rapidly if they know the study day will finish on completion of the meal. Food intake might also be influenced by the presence of others in the room, as eating in a sociable environment with other people has been seen to increase food intake (Herman & Polivy, 2005). Many of these problems can however be overcome with the use of careful planning. Long and Colleagues (Long *et al.*, 2002) informed the 23 men participating in their study that they could take home any food they did not eat during

the meal in an attempt to prevent the subjects from overeating. Masking the true aim of the study from participants is less likely to cause them to adjust their food intake in order to meet what they believe to be acceptable, for example Kral *et al*. (Kral *et al.*, 2004) informed subjects that the aim of their study was to investigate the effects of the intervention on food taste rather than the amount eaten. Experimenters have also instructed subjects to consume the buffet meal in isolated conditions (Himaya *et al.*, 1997;Hubert *et al.*, 1998) and informed them that they will remain in the laboratory until a set time *e.g.* 10.00 pm in order to avoid subjects requesting early meals, eating less and eating more quickly in the hope of leaving earlier (Himaya *et al.*, 1997).

1.12.3 Food diaries

In situations where food intake is to be monitored everyday within an environment outside of the laboratory, it is not always practical or even recommended to use direct methods like those discussed above and instead researchers might use food diaries. Here, subjects keep a daily record of everything they eat and drink either by weighing the foods, taking photos or by reporting portion sizes (Thompson & Byers, 1994). The data from these diaries are collected and analysed using computerised food databases. The length of recording food intake varies depending on the nature of the study but may last for 24 hours, a couple of days or up to a week (Thompson & Byers, 1994).

Using food diaries enables the researcher to consider the impact of varying environmental factors on feeding behaviour, however, as with the direct method above, food diaries do have limitations, the main one being underreporting. Healthy women during a seven day period, underreported their food intake by an average of 21% (Scagliusi *et al.*, 2003) and although a large volume of the literature focuses on the female population, there is also evidence that underreporting is a problem when using diet records in men (Goris *et al.*, 2000). Underreporting might be attributed to subjects consciously lying about their food intake, for example, not recording a bag of crisps or a chocolate bar that they have eaten because they don't want to be perceived as greedy or unhealthy by the experimenters (Scagliusi *et al.*, 2003). However, there can also be unconscious changes in food intake in situations when, without consciously deciding to do so, subjects change the foods that they eat in order to consume those that are easier to weigh and record (De Castro, 2000). Effective measures can be taken to ensure the impact of underreporting is minimal for example subjects might be instructed to eat their meals with another person present and this person will then be contacted by the research team to verify that the foods eaten and recorded in the diary were accurate and correct (De Castro, 2000).

1.12.4 Visual analogue scales

Visual analogue scales (VAS) are the most commonly used technique for measuring appetite (Mattes *et al.*, 2005). They are relatively easy to use and the 'pen and paper' style of test makes them relatively cheap to implement in a study. They also utilise an individual's ability to introspect providing accurate and individual insight into eating patterns and feeding behaviour (Mattes *et al.*, 2005).

Visual analogue scales comprise a series of questions related to the outcome variable. With each question comes a straight-line, the length of which differs but is typically between 100-150 mm long (Mattes *et al.*, 2005). At each end of the line is a statement providing the opposite responses to the question for example 'how hungry do you feel' is coupled with the statements 'I am not hungry at all' and 'I have never been more hungry'. Subjects read the question and place a mark through the line at the point which best matches their present feelings. The experimenter measures the distance, starting from the left, along the line to the mark and this distance is used as the appetite rating for that question (Flint *et al.*, 2000). Over time, a series of questions have been developed including 'How hungry do you feel?' 'How satisfied do you feel?' 'How full do you feel?' 'How much do you think you can eat?' 'Would you like to eat something sweet?' 'Would you like to eat something salty?' 'Would you like to eat something savoury?' 'Would you like to eat something fatty?' (Flint *et al.*, 2000), a combination of which are used to compile questionnaires. As well as appetite, VAS scales are also useful for determining other factors influencing food intake such as food palatability, food taste, food smell etc (Flint *et al.*, 2000). A limitation of their use, however, is that the components they measure are subjective and therefore vulnerable to change by internal and external factors (Flint *et al.*, 2000). Laboratory-based studies control for these factors but their impact may be more significant when VAS scales are used in the real world environment. Overall however, the validity and reliability of VAS scales for measuring appetite seems reasonable (Flint *et al.*, 2000;Porrini *et al.*, 1995;Stratton *et al.*, 1998) and they remain a popular method for use in research studies investigating appetite and feeding behaviour.

1.13 Physiological regulation of appetite and feeding behaviour

Many pathways are involved in the regulation of appetite and feeding behaviour, pathways which are both physiological and psychological. With regard to physiological control, two systems regulate food intake; short term control which is associated with the avoidance of over consumption at meal times and includes the release of appetite hormones and long

term control which preserves energy and fat stores within the body and involves the actions of leptin and insulin (Konturek *et al.*, 2005).

1.13.1 Gastrointestinal hormones

Gastrointestinal (GI) hormones are secreted from cells lining the gastrointestinal tract in response to ingested food and also secreted within the central nervous system where it is believed they transfer information between the GI tract and the brain (Strader & Woods, 2005). On reaching the brain, most GI hormones restrict food consumption and subsequently are referred to as satiety signals. One exception however is ghrelin, a GI hormone released from the stomach which stimulates appetite and increases food intake (Wren *et al.*, 2001). A review by Strader and Woods (Strader & Woods, 2005) suggested that at the time of publication there were 10 hormones believed to affect appetite. A summary of these hormones and their proposed effect on appetite can be seen in **Table 1.2.** Due to financial and methodological limitations, however, it is beyond the scope of this thesis to measure all GI hormones and therefore the focus will be on two current hormones of interest, ghrelin and leptin, which are described in detail below.

Table 1.2 Gastrointestinal hormones and expected effects on food intake

Hormone	Expected effect on food intake
Amylin	Decrease
Apolipoprotein A-IV	Decrease
Bombesin / Gastrin releasing peptide	Decrease
Cholecystokinin	Decrease
Enterostatin	Decrease
Gastric leptin	Decrease
Ghrelin	Increase
Glucagon-like peptide-1	Decrease
Oxyntomodulin	Decrease
Peptide tyrosine tyrosine	Decrease

Although ghrelin is not a true GI hormone, it does have an important role in the short term regulation of appetite and food intake (Strader & Woods, 2005;Wynne *et al.*, 2004). Ghrelin is a 28-amino acid peptide which has been modified by a *n*-octanoic acid, a modification that has proved essential for its role in regulating food intake (Ueno *et al.*, 2005;Wynne *et al.*, 2004). Ghrelin is predominantly synthesised and secreted from the stomach although it is also believed to be secreted from the duodenum, ileum, colon and

caecum (Wynne *et al.*, 2004) and there is further evidence that ghrelin might be synthesized within the brain (Kojima *et al.*, 1999). Ghrelin is orexigenic, meaning it stimulates rather than suppresses appetite and within the brain, it is thought that ghrelin targets neurons within the arcuate nucleus of the hypothalamus, stimulating the release of neuropeptide Y and Agouti-Related Peptide to help exert its orexigenic effect (Konturek *et al.*, 2005). Ghrelin concentrations gradually rise whilst fasting, reaching a peak just before food intake then rapidly returning to baseline concentrations, a pattern indicative of a role for ghrelin in the initiation of food intake (Cummings *et al.*, 2004). In nine healthy volunteers, Wren et al. (Wren *et al.*, 2001) showed ghrelin administration to increase energy intake by an average of 28%. A later study from Druce et al. (Druce *et al.*, 2005) investigated the effects of low and high doses of ghrelin on energy intake in lean and obese volunteers and they reported that a low dose of ghrelin increased energy intake in obese subjects whereas a high dose increased energy intake in lean and obese by 20% and 70%, respectively. In addition to the short-term regulation of food intake, there is also evidence of a long term role for ghrelin in regulating energy balance and body weight. Circulating concentrations of ghrelin correlate well with body weight (Wynne *et al.*, 2004) and furthermore, after significant weight loss in obese volunteers, ghrelin concentrations are reported to return to normal baseline concentrations (Cummings *et al.*, 2002).

One important consideration, however, is that total ghrelin comprises two separate forms, acylated and de-acylated ghrelin. De-acylated ghrelin is not believed to be a major regulator of appetite and it is acylated ghrelin that is suggested to exert a more direct effect (Broom *et al.*, 2007). In response to a mixed meal, acylated ghrelin concentrations are suppressed, a suppression which appears somewhat dependant on the macronutrient composition of the meal (Al Awar *et al.*, 2005;Blom *et al.*, 2006;Tannous dit *et al.*, 2006). In 11 healthy and normal weight young men and women, Al Awar *et al*. revealed a significant attenuation in acylated ghrelin concentrations after a meal with a balanced composition and a meal rich in protein (35% protein). The attenuation in ghrelin concentrations was however more prolonged following the high protein meal. Using a similar study design, Tannous dit et al. (Tannous dit *et al*., 2006) compared the effects of a high carbohydrate, high fat and high protein meal. They reported acylated ghrelin concentrations to be significantly lower following all three meals with a greater reduction following the high carbohydrate load. However, in support of the findings from Al Awar *et al*. (Al Awar *et al*., 2005), these authors also reported a longer lasting attenuation in acylated ghrelin following the high protein meal compared to carbohydrate and fat. Blom *et al*. (Blom *et al*., 2006) also reported a reduction in acylated ghrelin concentrations

following a high carbohydrate and a high protein meal, however they failed to report any difference between these responses. It does appear, therefore, that the presence of different macronutrients within the diet might have valuable implications for attenuating acylated ghrelin concentrations. It also appears that the response time of total ghrelin and acylated ghrelin to food intake may vary as, compared to total ghrelin, acylated ghrelin appears to respond more quickly to glucose consumption. In contrast to total ghrelin, the literature investigating acylated and de-acylated ghrelin in their own separate forms appears limited and there is a need to gain further understanding of how acylated ghrelin might mediate appetite and how it might be regulated by interventions such as exercise.

1.13.2 Leptin and insulin

Leptin a 167-amino acid peptide hormone secreted from white adipose tissue was first identified in 1994 (Zhang *et al.*, 1994) and is thought to contribute to the long term regulation of energy balance and body mass (Friedman & Halaas, 1998;Klok *et al.*, 2007). Leptin is believed to exert its effect via the inhibition of orexigenic neurones within the hypothalamus, namely neuropeptide Y and Agouti-Related Peptide whilst also activating anorexigenic neurons (Konturek *et al.*, 2005). Widely considered as an 'anti-starvation' hormone, in times of low or restricted energy availability, leptin suppresses excessive energy expenditure helping to preserve energy stores (Flier, 1998) but under normal conditions of energy availability, leptin administration has been shown to lower body mass, increase metabolic rate and to increase activity levels, at least in rodents (Halaas *et al.*, 1995). In humans, there is evidence that leptin administration increases muscular fat oxidation (Dyck, 2005) and when leptin deficiency is treated with leptin, the observed effects have included decreased appetite, weight loss and greater levels of physical activity (Licinio *et al.*, 2004). Compared to rodents however, the ability of leptin to alter human energy expenditure remains unclear (Klok *et al.*, 2007). As well as these direct effects, leptin may also regulate body weight indirectly, via its inhibitory effect on total ghrelin (Konturek *et al.*, 2005). Considering the role for leptin in regulating body mass, it is somewhat surprising therefore, that circulating concentrations of leptin are elevated in obese individuals compared to their normal weight peers (Considine *et al.*, 1996) but fail to induce lower body weights. Subsequently, it has been suggested that those who are obese and overweight suffer some degree of leptin resistance (Dyck, 2005) preventing it from exerting its normal metabolic effects.

Although a major site of leptin synthesis and secretion is adipose tissue, leptin is also secreted from the stomach as gastric leptin a form which may contribute up to 25% of total

circulating leptin concentrations (Strader & Woods, 2005). Like total leptin, gastric leptin is believed to be regulated in part by states of energy availability. Gastric leptin also interacts with cholecystokinin (CCK); a greater synthesis of gastric leptin occurring in the presence of CCK (Strader & Woods, 2005) whilst an increase in insulin might also augment the secretion of gastric leptin from the intestine (Strader & Woods, 2005). Although the effects of adipose derived leptin have been extensively investigated, the effects of gastric leptin *per se* have not been clarified, however it is likely that both forms of leptin have a role in regulating energy balance and body mass (Strader & Woods, 2005).

Insulin appears to exert both long- and short-term regulatory effects on appetite and energy balance, effects which might be mediated by its role as an adiposity signal. The rate of insulin secretion is proportional to the degree of adiposity for an individual and a change in body weight induces a change in insulin secretion (Porte, Jr. & Woods, 1981). As well as plasma insulin, insulin secretion within the cerebrospinal fluid may also act as an adiposity signal; in obese individuals, cerebrospinal fluid concentrations of insulin are elevated when compared to lean controls, and decline with fasting (Porte, Jr. & Woods, 1981). The effects of insulin on food intake have been investigated using both animal and human models. In a relatively early study, Ikeda *et al*. (Ikeda *et al.*, 1986), infusing insulin directly into the brain of lean and obese Zucker rats, reported a decline in food intake and body mass but in lean rats only. In baboons, a prolonged infusion of insulin also caused a decrease in food intake and body weight whereas removing the insulin stimulus saw both food intake and body weight return to baseline (Porte, Jr. & Woods, 1981). In humans, insulin has been shown to regulate appetite and food intake. Fasting insulin concentrations and the postprandial insulin response correlate well with food intake at a subsequent ad libitum buffet meal in lean individuals (Verdich *et al.*, 2001) and Flint *et al*. (Flint *et al.*, 2006) reported that insulin was significantly and positively related to postprandial feelings of fullness in young men. Interestingly however, it appears that the relationship between insulin, appetite and food intake may be disturbed in overweight and obese individuals and compared to their lean peers, those who are overweight fail to show any relationship between insulin and appetite or food intake (Flint *et al.*, 2007;Verdich *et al.*, 2001); a decrease in insulin sensitivity may go someway to explain these differences (Flint *et al.*, 2007;Verdich *et al.*, 2001). Insulin may regulate appetite via a direct effect on the hypothalamus in the brain (Verdich *et al.*, 2001) but it might also interact with other satiety hormones such as peptide tyrosine-tyrosine 3-36, glucagon like peptide-1 and CCK in order to regulate appetite and food intake (Flint *et al.*, 2007).

1.14 Behavioural regulation of appetite

As well as physiological mechanisms there are a number of behavioural and cognitive traits that also act to regulate food intake. Rather than eat the amount and type of food that they would like to, some individuals exert strict control over their daily food intake, a process referred to as cognitive restraint (De Castro, 1995). Those who report higher levels of restraint typically consume lower amounts of food; a relationship observed in adolescents (Wardle *et al.*, 1992), women (Bellisle & Dalix, 2001;De Castro, 1995) and men (De Castro, 1995). With regard to their eating patterns, those who are more restrained may eat smaller meals but do not appear to reduce their meal frequency (De Castro, 1995). With regard to macronutrient preference, a smaller food intake is reflected in lower quantities of fat and carbohydrate being consumed (De Castro, 1995) with restrained eaters consuming lower quantities of high fat foods such as cheese, fish and red meat but greater amounts of fruit, vegetables (Beiseigel & Nickols-Richardson, 2004;De Castro, 1995), chicken (De Castro, 1995) and low fat foods (Rideout *et al.*, 2004). The reasoning for controlling food intake may differ between genders where for women there is a 'fear of weight gain' whilst men appear to restrict food intake for the purposes of weight loss (De Castro, 1995). In contrast to cognitive restraint, different extremes of disinhibition may also affect food intake. Studies have shown those with greater levels of disinhibition to suffer a higher risk of weight gain and a higher BMI than those with lower levels (Dykes *et al.*, 2004;Hays *et al.*, 2002) and higher levels of disinhibition reportedly increase food intake at a meal in young lean women (Westenhoefer *et al.*, 1994). Lindroos et al. (Lindroos *et al.*, 1997) also reported larger energy intakes at a meal in obese women with a higher level of disinhibition but failed to observe the same relationship between disinhibition and food intake in normal weight controls.

In addition to behavioural control, social influences also have implications for feeding behaviour. Herman and Polivy (Herman & Polivy, 2005) suggest that in the presence of others, people use the food intake of those around them to determine how much is acceptable for them to eat. The literature suggests that in the presence of others, an individual is likely to consume more food than when alone (De Castro, 1995;De Castro & Brewer, 1992) and people might eat up to 75% larger meals in a group environment when compared to eating alone (De Castro & Brewer, 1992). It is however not only the mere presence of other people but also the gender and relationships between those who are sharing a meal that effect food consumption. Women who eat in the company of other women consume less food than when they are in the presence of another man (De Castro,

1994) and when eating in the presence of friends and family, people are likely to eat more than when accompanied by others, such as work colleagues (De Castro, 1994).

There is clear evidence that appetite and feeding behaviour can be regulated via several different mechanisms, mechanisms that are both physiological and behavioural. It is likely that interactions between a number of these mechanisms will determine the overall effect on food intake and, therefore, it is important to consider how these factors may interact when trying to accurately measure the effect of different intervention strategies on appetite regulation.

1.15 The role for exercise in appetite regulation

Those who regularly exercise are better able to prevent weight gain and maintain stable body weights compared with their sedentary peers (Wareham *et al.*, 2005). It is possible that exercise induces changes in, and a tighter coupling between, exercise, appetite and food intake and there are a number of pathways by which such an effect might occur.

1.15.1 Exercise and leptin

A number of studies have investigated the training effect on fasting plasma leptin concentrations using exercise programmes ranging from 12 weeks to 16 months (Frank *et al.*, 2005;Hickey *et al.*, 1997;Okazaki *et al.*, 1999;Pasman *et al.*, 1998;Perusse *et al.*, 1997;Reseland *et al.*, 2001). Plasma leptin concentrations were 35% lower following sixteen months of aerobic training in obese men (Pasman *et al.*, 1998) and 39% lower after 12 weeks of training in obese women (Okazaki *et al.*, 1999). Similar results have been reported in many other studies although the magnitude of the effect varies with some reporting leptin to be reduced by as much as 39% (Pasman *et al.*, 1998) whilst others report just a 7% reduction (Frank *et al.*, 2005). Furthermore, it also appears that exercise might have a lowering effect on leptin that occurs without any change in body fat (Hickey *et al.*, 1997;Pasman *et al.*, 1998). Despite consistent data showing prolonged training to attenuate plasma leptin, it appears that a gender effect may occur. Hickey et al. reported an 18% decline in leptin concentrations in women following 12 weeks training but no effect was evident in male subjects (Hickey *et al.*, 1997). In contrast, Perusse et al. reported a 14% decline in leptin concentrations in men but no effect was seen in women (Perusse *et al.*, 1997). It is possible that methodological differences and variation in subject number may explain these conflicting results but there remains a need to clarify whether there is a true gender effect in the exercise-induced lowering of leptin.

In contrast to training studies, the effects of a single session of exercise on leptin are less clear. It has been suggested that an exercise-induced attenuation of plasma leptin concentrations is dependant on the energy expended during the activity. Studies have shown that plasma leptin remains unchanged following 60 minutes of cycling at 50% $\dot{V}O_2$max (Racette *et al.*, 1997), 60 minutes of treadmill running at 50-60% $\dot{V}O_2$max (Kyriazis *et al.*, 2007) and prolonged treadmill running at 70% $\dot{V}O_2$max (Essig *et al.*, 2000). However, interventions inducing much larger energy expenditures such as a 101 mile marathon race (Landt *et al.*, 1997) or a 100 km ultramarathon (Zaccaria *et al.*, 2002) reduced postprandial leptin concentrations by 32% and 71%, respectively. Three hours of aerobic cycling at 60% $\dot{V}O_2$max (Keller *et al.*, 2005) and two hours of treadmill running at 75% $\dot{V}O_2$max (Tuominen *et al.*, 1997) were also shown to lower fasting plasma leptin concentrations by 50% and 34%, respectively. Therefore it does appear that larger energy expenditures associated with exercise are required to induce lower leptin concentrations. However, within these studies the subject cohorts were very different ranging from obese men (Kyriazis *et al.*, 2007;Racette *et al.*, 1997) to trained athletes (Landt *et al.*, 1997;Zaccaria *et al.*, 2002) and these differences may themselves explain some of the variation observed in the literature. Furthermore, the use of a single measure of leptin taken immediately post-exercise may overlook any change in leptin that occurs after a delay of some hours (Essig *et al.*, 2000) and indeed, researchers have reported lower leptin concentrations 5 hours (Keller *et al.*, 2005), 44 hours (Tuominen *et al.*, 1997) and 48 hours (Essig *et al.*, 2000) post-exercise.

As discussed above, it has been suggested that lower leptin concentrations may be determined by the exercise-induced energy expenditure and thus changes in energy balance status. However, Van Aggel-Leijssen *et al*. (Aggel-Leijssen *et al.*, 1999) measured 24 hour leptin profiles in response to exercise either with or without a state of energy balance. Their data suggested that exercise in energy balance lowered 24 hour plasma leptin by 20% compared to rest suggesting that exercise *per se* rather than associated changes in energy balance might attenuate leptin concentrations. In contrast, however, Hilton and Loucks (Hilton & Loucks, 2000) reported exercise performed in a state of energy balance to have no effect on leptin compared to exercise with an energy deficit. It therefore remains somewhat unclear as to what extent the energy expended during exercise and the associated energy deficit may mediate lower plasma leptin concentrations. It is possible that rather than energy balance status alone, leptin responds to exercise-induced changes in energy substrate balances, specifically changes in carbohydrate and fat availability that

occur following the utilisation of such fuel sources during exercise (Aggel-Leijssen *et al.*, 1999;Hilton & Loucks, 2000). Changes in plasma insulin might also mediate lower leptin concentrations as insulin stimulates leptin secretion (Saad *et al.*, 1998) and therefore, lower insulin responses following exercise might inhibit the synthesis and or release of leptin from adipocytes. Findings from Essig et al. (Essig *et al.*, 2000) of a decline in insulin concentrations that preceded lower leptin measured 24 hours post exercise do indeed support a role for insulin in mediating leptin.

1.15.2 Exercise and ghrelin

Following three months of training, fasting total ghrelin concentrations appear to increase, but only when a reduction in body mass is observed (Leidy *et al.*, 2004). Similar findings were reported following a 12 month training programme where a 5% increase in fasting total ghrelin was related to an associated reduction in body mass (Foster-Schubert *et al.*, 2005). These data suggest that the effects of prolonged exercise on total ghrelin may be dependant on, and sensitive to, changes in energy availability and body mass. It is unclear why prolonged exercise might increase total ghrelin concentrations although considering its orexigenic properties (Druce *et al.*, 2005;Wren *et al.*, 2001) one possible explanation is that following prolonged training, where energy stores become depleted and subsequently body mass is lost, ghrelin stimulates appetite and energy intake to preserve body weight.

A number of studies have investigated the effects of acute exercise on both fasting and postprandial plasma total ghrelin concentrations. A 60 minute treadmill run at 75% $\dot{V}O_2$max in 18 healthy volunteers (Burns *et al.*, 2007) and a 60 minute treadmill run at 60% $\dot{V}O_2$max in obese men (Kyriazis *et al.*, 2007) had no effect on fasting total ghrelin concentrations. A 60 minute cycling intervention at 65% HRmax in 12 men and women (Martins *et al.*, 2007a) also failed to have any effect this time on postprandial total ghrelin concentrations. These studies are only a few of those investigating the effects of acute exercise on total ghrelin and a number of others also report no change in total ghrelin post-exercise (Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Schmidt *et al.*, 2004;Zoladz *et al.*, 2005). Recently however, two studies have observed exercise-induced changes in total ghrelin. Following maximal rowing, Jurimae *et al*. (Jurimae *et al.*, 2007b) reported ghrelin to be increased immediately post-exercise, although the effect did not persist beyond 30 minutes of recovery. Vestergaard *et al*. (Vestergaard *et al.*, 2007), however, reported in healthy subjects, following a maximal exercise test, fasting total ghrelin concentrations to be attenuated for up to an hour post-exercise. It is not clear why these authors found changes in total ghrelin concentrations, but it should be considered that both studies failed

to use a control trial making it difficult to draw any firm conclusions about whether these exercise-induced changes in ghrelin were a true effect. Together, findings from acute studies suggest that total ghrelin is not affected by exercise. However none of the above studies considered the separate components of total ghrelin i.e. the acylated and de-acylated, forms in their analyses. Although exercise does not appear to change total ghrelin concentrations, it might alter the ratio of acylated to de-acylated ghrelin and subsequently this could be a mechanism by which exercise mediates appetite and feeding behaviour. Mackelvie *et al*. (Mackelvie *et al.*, 2007) measured total and acylated ghrelin concentrations following five consecutive days of moderate exercise in normal and overweight subjects. In the absence of any change in total ghrelin concentrations, the authors reported the acylated ghrelin response to be significantly higher following exercise with a greater increase in the normal weight compared to overweight subjects. In contrast however, Broom *et al*. (Broom *et al.*, 2007) reported that in healthy young men, postprandial acylated ghrelin concentrations following a 60 minute treadmill run at 75% $\dot{V}O_2$max were 35% lower compared to a control trial. It is difficult to explain the differences in their findings, although Mackelvie *et al*. (Mackelvie *et al.*, 2007) did implement five consecutive days of exercise compared to the single one hour session used by Broom *et al*. (Broom *et al.*, 2007). The effect of five days of training on increasing acylated ghrelin is consistent with studies showing an increase in total ghrelin concentrations following prolonged training, thus it is possible that prolonged exercise and a single session of exercise have different effects on acylated ghrelin concentrations. At present however, the literature investigating the associations between exercise and acylated ghrelin is limited and there is clearly a need for more research investigating the effects of exercise on acylated ghrelin, both in healthy and diseased populations.

1.15.3 Exercise and subjective ratings of appetite

With regard to the effects of training *per se* on appetite, limited information is available. A 48-week exercise and dietary intervention programme failed to have any effect on hunger ratings in obese women compared to a dietary intervention alone (Wadden *et al.*, 1997). Similarly, a six week moderate intensity exercise programme had no effect on ratings of hunger or fullness in a group of healthy men and women (Martins *et al.*, 2007b). A study of habitual exercisers by Long *et al*. (Long *et al.*, 2002) did show fasting hunger scores to be significantly lower compared to those who were inactive, although no differences in appetite between the two groups were observed in the postprandial state. It seems therefore that prolonged training has a minimal effect on regulating appetite.

The effect of acute exercise on subjective ratings of appetite is however less clear. Feelings of hunger were decreased and satiety increased immediately following a 20-minute brisk walk in obese women with satiety remaining elevated up to one hour post-exercise (Tsofliou *et al.*, 2003). Hunger was also suppressed immediately after exercise in 12 young and healthy men and women (Martins *et al.*, 2007a) and Westerterp-Plantenga *et al*. (Westerterp-Plantenga *et al.*, 1997a) reported lower hunger scores in obese and non obese men 10 minutes after prolonged cycling. A recent study has also shown that following a 60 minute treadmill run, hunger was suppressed for one hour on completion of exercise (Burns *et al.*, 2007).

Although many studies have observed an effect of exercise on appetite, others fail to show such a response. An immediate suppression in hunger was observed by Martins *et al*. (Martins *et al.*, 2007a), however, the effect was transient and was no longer evident one hour later. Furthermore, they also failed to report any effect of exercise on feelings of fullness or motivation to eat (Martins *et al.*, 2007a). Similarly, no effect of exercise on fullness or desire to eat (Lluch *et al.*, 2000;Pomerleau *et al.*, 2004) and hunger or prospective food consumption (Pomerleau *et al.*, 2004) measured during a prolonged period after low or high intensity exercise (Pomerleau *et al.*, 2004) or forty minutes of cycling (Lluch *et al.*, 2000) has been observed.

Such equivocal findings with regard to the effect of exercise on appetite might be explained by different subject groups i.e. male or female, obese or non obese, as appetite regulation might differ between different populations (Blundell *et al.*, 2003;King *et al.*, 1997b). It is also possible that a low subject number may have limited the statistical power to uncover any exercise effect (Flint *et al.*, 2000). At this time, the role for acute exercise in appetite regulation is equivocal and requires further investigation. Furthermore, there is a need to investigate what impact exercise, coupled with a state of energy balance, might have on appetite regulation, and by doing this, further insight might be gained into how people who regularly exercise and maintain stable body weights differ compared to their sedentary peers.

1.15.4 Exercise and food intake

From their study of habitual exercisers, Long *et al*. (Long *et al.*, 2002) reported that following a meal of either high or low energy content, regular exercisers ate significantly less food at a subsequent buffet meal compared with non exercisers. Martins *et al.* (Martins *et al.*, 2007b) also reported energy intake at a buffet meal to be significantly

lower when the meal was preceded by a high energy load compared to a low energy load measured after a six week training programme. These findings suggested that those who exercised were better able to compensate for previous energy loads by adjusting subsequent food intake compared to non-exercisers. There is also evidence that during prolonged periods of training, people gradually increase their energy intake to meet the energy demands of the activity, thus facilitating a state of energy balance and stable body weights (Blundell *et al.*, 2003). The ability of acute exercise to modify food intake does, however, appear less conclusive. Energy intake in obese subjects was shown to be lower when measured immediately after a two hour cycling intervention (Westerterp-Plantenga *et al.*, 1997a). Conversely, Martins *et al*. (Martins *et al.*, 2007a) reported absolute energy intake at a buffet meal to increase following exercise compared to rest, although when they considered the amount of energy expended during exercise itself by calculating relative energy intake, they reported energy intake was actually lower. Using a similar method, Pomerleau et al. also reported relative energy intake to be lower following low and high intensity exercise interventions (Pomerleau *et al.*, 2004). Despite these findings however, there remains controversy and studies using a variety of interventions such as medium and high volumes of cycling (Hubert *et al.*, 1998;Stubbs *et al.*, 2002a;Thompson *et al.*, 1988), brisk walking (Tsofliou *et al.*, 2003) and treadmill running (King *et al.*, 1997a) have all shown no change in subsequent energy intake. In fact, Stubbs *et al*. (Stubbs *et al.*, 2002b) reported that following high volume exercise women increased their food intake to compensate for approximately 33% of the energy expended during the exercise intervention.

During exercise energy substrate stores are depleted, a possible response to which might be a change in macronutrient intake post-exercise, facilitating the replenishment of fuel stores (Bellisle, 1999). Women shown to compensate for the energy expended during exercise have done so by increasing fat and carbohydrate intake (Stubbs *et al.*, 2002b). Obese and non-obese men have been reported to favour carbohydrate rather than fat at a buffet meal consumed post-exercise (Westerterp-Plantenga *et al.*, 1997a) whereas active women consumed more fat and protein than carbohydrate following high intensity exercise (Pomerleau *et al.*, 2004). In response to an hour of exercise however, Martins *et al*. (Martins *et al.*, 2007a) reported no change in macronutrient consumption in 12 (6 men, 6 women) healthy, normal weight volunteers, findings consistent with those from earlier studies (King *et al.*, 1997a;Stubbs *et al.*, 2002a;Tsofliou *et al.*, 2003). Thus the ability of exercise to alter macronutrient preferences remains unclear.

Whilst these studies investigating the effect of exercise on food intake are generally well designed, they vary in their design including the subject population, exercise intervention, length of time between exercise and buffet meal and duration over which food intake is monitored. All of these variables are likely to introduce variation in their data and may explain the different findings. Furthermore, to the author's knowledge, and similar to the research investigating exercise and appetite ratings, no attempt has been made to consider the role for an energy balance state in regulating food intake post-exercise and this warrants further investigation to understand how regular exercise might induce tighter coupling between energy intake and expenditure.

1.16 A problem with exercise!

Exercise and training are common strategies implemented for weight loss, particularly in overweight and obese populations. However, a common problem arises where people struggle to induce any initial weight loss or re-gain the weight that they have lost. Different compensatory responses to exercise have now been identified that might explain why some individuals find weight loss following exercise challenging (King *et al.*, 2007). King *et al*. (King *et al.*, 2007) classified these compensatory responses as metabolic or behavioural changes with behavioural changes likely to be a greater barrier to exercise-induced weight loss. Such compensatory responses might also be 'automatic' or 'volitional' reflecting the amount of control the individual exerts over a particular response.

Increasing food intake in response to regular exercise will eliminate any exercise-induced energy deficit, thus inhibiting weight loss. A greater food intake may manifest itself by increasing meal or portion sizes or by increasing the number of eating episodes or snacks consumed after exercise (King *et al.*, 2007). Food choice might also change with a preference for more energy dense foods, a change which may be a result of individuals feeling more justified in having 'food rewards' because of the calories they have expended during the activity (Blundell *et al.*, 2003). A problem with this notion of rewarding yourself for exercise is that people tend to overestimate how much energy they have expended or underestimate the energy content of a particular food, subsequently leading to excess calories being consumed compared to those utilised (Blundell *et al.*, 2003). The greater appeal of food post-exercise and the pleasure actually derived from eating might also be enhanced (Lluch *et al.*, 1998), factors which again can potentially lead to greater food intakes.

It is important to consider, however, that there are two components regulating energy balance, energy expenditure and energy intake. Some individuals may not alter their energy intake following exercise but rather may lower their energy expenditure. Stubbs *et al*. (Stubbs *et al.*, 2004) measured energy intake and expenditure during seven days of exercise and reported that a gradual return to energy balance was evident during this time, although no increase in energy intake was observed. Measurement of the non-exercise energy expenditure revealed that exercise reduced the energy cost of daily living, suggesting subjects became less active outside of structured exercise; a possible cause of this compensatory response might be increased levels of fatigue preventing the maintenance of normal non-exercise activity levels (King *et al.*, 2007;Stubbs *et al.*, 2004). It is important that the impact of these compensatory responses to exercise be considered when prescribing exercise for weight loss and a greater understanding of how appetite and feeding behaviour might change during the hours and days following exercise might provide insight into how acute exercise can successfully overcome such compensatory responses.

1.17 Summary

The aim of this chapter was to provide the reader with insight and understanding into postprandial metabolism, appetite regulation and feeding behaviour and the role for acute exercise in mediating such factors. Cardiovascular disease and obesity are both major health concerns for the UK and there is a need to understand how such diseases can be prevented and treated. Metabolic perturbations within the postprandial state promote the development and progression of atherosclerosis and acute exercise is a successful intervention for mediating such postprandial fluctuations. It remains unclear however to what extent these effects are mediated by exercise *per se* or the associated energy deficit. Regular exercisers seem better able to prevent weight gain and maintain stable body weights suggestive of a prolonged energy balance state. Investigating the metabolic, appetite and feeding responses to acute exercise, coupled with a state of energy balance, might provide further insight into how those who exercise induce tighter coupling between energy intake and energy expenditure.

The aim of this thesis and the following experimental chapters is to further knowledge into how acute exercise might affect postprandial metabolism, appetite control and feeding behaviour with an emphasis on understanding the role an energy balance state might have on mediating such effects. Chapter 3 investigates the effects of moderate intensity exercise, both with and without the associated energy expenditure replaced, on

postprandial metabolism, including lipaemia, insulinaemia, glycaemia and energy substrate utilisation, measured the following day. Chapter 4 extends this investigation by examining the effects of exercise, with and without energy replacement, on PWV an index of endothelial function. This chapter also aimed to identify what factors might contribute to any exercise-induced change in PWV, including TG and asymmetrical dimethylarginine (ADMA) concentrations. Chapter 5 determines the effects of exercise, with and without energy replacement, performed the previous afternoon, on markers of appetite and circulating concentrations of the appetite related hormones leptin and total ghrelin. Chapters 6 & 7 are a progression of the earlier chapters and examine the immediate effects of a state of energy balance with high (exercise) or low (rest) energy turnover. Chapter 6 focuses on postprandial metabolism, in particular lipaemia, insulinaemia and glycaemia and whole body fat oxidation. Chapter 7 however, investigates appetite regulation including markers of appetite, food palatability, energy and macronutrient intakes and circulating concentrations of total and acylated ghrelin hormones.

CHAPTER 2
GENERAL METHODS

This chapter provides a description of all general methods that have been implemented in the following experimental chapters. Methods specific to individual chapters will be highlighted as such. Methods used for statistical and data analysis are not reported here but rather are described separately in each experimental chapter. Ethical approval for all research studies was sought from and provided by the North Glasgow University Hospitals NHS Trust Ethics Committee (Chapters 3, 4 and 5) and the Faculty of Biomedical and Life Sciences Ethics Committee at Glasgow University (Chapters 6 and 7).

2.1 Subject recruitment and screening

All subjects resided in the Glasgow area and were recruited via local advertising and an advert placed on the website of Glasgow University's Sport and Recreation Service. Prior to participation all subjects attended the university for a screening visit. An information sheet was provided describing the aim of the study, the experimental procedures involved and any potential risk or discomfort associated with these procedures. Written, informed consent was recorded for each subject (**Appendix 1A**). Questionnaires detailing the subject's past and present health status and family history of disease were completed (**Appendix 1B**) as were physical activity questionnaires providing a record of their weekly exercise routine (**Appendix 1C**). Resting blood pressure was measured using an automated sphygmomanometer (Omron Healthcare, Inc., Illinois, USA) and fasting blood samples were taken to ensure subjects met the inclusion criteria for each study. The following criteria were used for subject recruitment:

i. Male aged 30 – 60 years (Chapters 3, 4 & 5)
ii. Pre-menopausal female aged 18 – 45 years (Chapters 6 & 7)
iii. BMI of 30 – 40 $kg.m^{-2}$ (Chapters 3, 4 & 5) and 25 – 40 $kg.m^{-2}$ (Chapters 6 & 7)
iv. Waist circumference over 94 cm (~ 38 inches) (Chapters 3,4 & 5)
v. Blood pressure below 150 / 90 mmHg
vi. Plasma glucose concentration < 7.0 $mmol.l^{-1}$ and no known diabetes
vii. Normal liver function (Chapters 3, 4 & 5)
viii. Non-smoking
ix. Not taking any medication known to interfere with lipid or energy substrate metabolism

Power calculations, based on data for the reproducibility of TG and insulin responses to an oral fat tolerance test with standard deviations of 9% and 19%, respectively, (Gill *et al.,* 2005) revealed that nine subjects would be needed to detect the expected change in postprandial TG (9%) and 15 subjects would be needed to detect the expected change in postprandial insulin (15%) with 80% power at $\alpha = 0.05$. Previous studies have indicated that 8-12 subjects are sufficient to show a significant effect of prior moderate intensity exercise on postprandial TG, insulin and NEFA concentrations and on postprandial fat oxidation (Gill & Hardman, 2000; Gill *et al.,* 2001; Gill *et al.*, 2002a). In chapters 3, 4 and 5, 15 subjects were recruited, however, one subject was removed from the study for health reasons and one withdrew for personal reasons. In chapters 6 and 7, 14 subjects were recruited but one withdrew, again, due to personal reasons.

2.2 Exercise stress test

Each subject participating in the studies described in Chapters 3, 4 and 5 completed an exercise electrocardiogram (ECG) stress test to ensure that they did not possess any cardiovascular contraindications to participate. All stress tests were completed by a qualified medical practitioner using a modified version of the Bruce protocol exercise ECG test (Bruce *et al.*, 1973). A 12-lead electrode configuration was used and remained in place at rest, during exercise and for a short post-exercise recovery period, throughout which time continual ECG traces and heart rate measurements were recorded. Blood pressure was measured at rest prior to the test and then on completion, five and 10 minutes after exercise. One subject had to be excluded from the study due to an irregular ECG trace. The volunteer was informed about the findings and a letter was sent directly to their general practitioner providing further details and instruction for future referral and treatment.

2.3 Anthropometric measurements

The techniques described below were used for anthropometric measurements in all experimental chapters.

2.3.1 Height

Height was measured using a Stadiometer (Invicta Plastics Ltd, Leicester, UK). Subjects stood barefoot, with their back against a fixed backboard and their arms hanging laterally by their body. The head was positioned with the line of eyesight perpendicular to the backboard. Subjects were instructed to relax and a moveable headboard was lowered to

the top of the head with light pressure added to compress the hair. Gentle upward pressure was applied to the lower jaw and height was measured to the nearest 0.01 metre.

2.3.2 Body Mass

Body mass was measured using a manual balance weighing scale (Avery, Birmingham, UK), to the nearest 0.05 kg. All subjects were weighed in private wearing minimal clothing, typically lightweight shorts and a t-shirt, whist standing with both feet flat on the balance and their arms hanging laterally by their body. All footwear, substantial jewellery and accessories, e.g. watches, and items within pockets were removed prior to any measurement. Body mass was measured using the same balance scales throughout all experimental studies.

2.3.3 Skinfold Measurement

Skinfold measurements were made at the bicep, tricep, subscapular and suprailiac sites, on the right hand side of the body, with the subject stood in the standard anatomical position. Skinfold thickness was measured to the nearest 0.1 mm using Harpenden skinfold calipers (Cranlea and Company, Birmingham, UK). The same set of calipers was used throughout all experimental chapters and measurements were taken by the same experimenter to limit intra-observer variability. To avoid any unnecessary compression of the adipose tissue, and subsequent underestimation of skinfold thickness, a single reading was taken, approximately three seconds after the caliper jaws were released, at each of the four measurements sites and then repeated a further two times. An average of each of the three readings was used for analysis. Skinfold measurement sites were located using the following criteria; *bicep*: mid-position between the acromial process and inferior olecranon at the most anterior aspect of the arm, with the forearm supinated, *tricep*: mid-position between the acromial process and inferior olecranon at the most posterior aspect of the arm, with the forearm supinated, *subscapular*: the inferior angle of the scapular taking a skinfold along the natural cleavage line of the skin, *suprailiac*: immediately superior to the iliac crest on the most lateral aspect of the mid-axillary line of the body. A summary measure of each subject's body composition was calculated using the sum of the four skinfolds.

2.3.4 Circumference measurement

Waist and hip circumferences were measured using a non-elastic measuring tape, with the subjects stood in the standard anatomical position, wearing minimal clothing, typically a lightweight pair of shorts and t-shirt. The tape measure was placed around the

measurement site avoiding any compression of the adipose tissue. All measurements were taken at the end of a normal expiration, and a total of three measurements were made at each site with the average used in further calculations. Waist and hip circumferences were measured by the same experimenter throughout all experimental chapters to limit any intra-observer variability. *Waist circumference* was measured at the narrowest part of the torso or, if this could not be identified, at the level midway between the iliac crest and costal border. *Hip circumference* was measured at the level where there was greatest extension of the buttocks, approximately at the level of the pubic symphisis. Waist circumference was divided by hip circumference to determine the waist to hip ratio.

2.4 Expired air measurements

Expired air collections were made using two separate techniques described in detail below. The ventilated hood method was used for measurement of metabolic rate both at rest in preliminary sessions and throughout all postprandial observation periods. The use of Douglas bags was implemented during all preliminary exercise tests and during experimental trial treadmill walks.

2.4.1 Measurement of resting metabolic rate

Resting metabolic rate (RMR) and energy substrate utilisation were measured in each subject after an overnight fast of at least twelve hours. Subjects attended the metabolic suite where they were instructed to make themselves comfortable and then, when ready, to lie supine on the couch, with their head resting on the pillow provided. After a 10-minute rest, the ventilated hood, connected to the Deltatrac monitor (Deltatrac Metabolic Monitor, Datex Engstrom, Kent, UK) was placed over the subjects head, with the surrounding 'skirt' secured in place. Another 10-minute acclimatisation period was completed to familiarise subjects with the hood and to ensure a resting state was achieved. During the measurement period, air flowed continuously into the hood, over the subjects' head, whilst expired air was extracted at the same rate into the body of the Deltatrac. Here the expired fraction of oxygen and carbon dioxide were determined and oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were calculated. During preliminary tests, expired air measurements were made continuously for 25 minutes, providing minute by minute values for $\dot{V}O_2$ and $\dot{V}CO_2$ and an average of the final 20 minutes of data was used for the calculation of non-protein respiratory exchange ratio, energy expenditure and rate of energy substrate utilisation using indirect calorimetry as described below. For all postprandial metabolic assessment periods, expired air collections were collected for 15 minutes with an average of the final 10 minutes used in further calculations.

Prior to its use, the Deltatrac was calibrated using reference gasses (QUICK CAL™ Calibration Gas, Datex-Ohmeda Division, Helsinki, Finland) of a known oxygen (95%) and carbon dioxide (5%) content. Further corrections were made for barometric pressure. For the duration of all measurements, preliminary tests and experimental trials, the metabolic suite was quiet and kept at a constant room temperature of 24°C.

2.4.1.1 Calculation of daily energy requirements

To calculate daily energy expenditure for each subject, their RMR was multiplied by a physical activity level (PAL). In Chapters 3, 4 and 5 a PAL of 1.55 was applied, the equivalent activity level of a sedentary male (FAO/WHO/UNU, 1985), and the estimated daily energy expenditure was used to determine the energy content of the pre-trial control diet. In Chapters 6 and 7, a PAL of 1.20 was applied, the equivalent activity level of an individual who is predominantly chair or bed bound (Shetty, 2005), and the energy expenditure during the six-hour postprandial metabolic assessment period, on which the energy content of the test meal was based, was calculated as 6 x RMR x 1.2.

2.4.2 Measurement of oxygen consumption and carbon dioxide production during exercise

At rest, prior to exercise, during all exercise sessions and during immediate post-exercise recovery measurements, Douglas bags were used to collect expired air samples.
Prior to all resting measurements, subjects completed a five-minute run in period to ensure they were comfortable with the equipment and in a true resting state. Expired air samples were collected into 100 or 150 litre Douglas bags (Cranlea and Company, Birmingham, UK). Throughout all measurements, subjects wore a nose clip, breathing through a mouth piece connected to a valve which directed the flow of expired air along a length of corrugated, plastic tubing connected to the Douglas bag via another two-way valve, the use of which controlled the flow of expired air into the Douglas bag.

Once expired air had been collected, a small quantity of gas was extracted from the bag at a constant flow rate measured by a flow meter. The extracted air was passed into a gas analyser (Servomex 4000 Series, Servomex Group Limited, E.Sussex, UK) where the percentage fraction of oxygen and carbon dioxide in each separate expired air sample was measured. The remaining volume of air in each Douglas bag was extracted, using a vacuum, through a dry gas meter (Harvard Apparatus, Ltd., Kent, UK). The temperature of the air was measured using a thermometer housed within the dry gas meter.

Prior to any expired air measurements, all Douglas bags were evacuated to remove any residual air. The gas analyser was calibrated before use against reference gases (BOC Gases, BOC Limited, Surrey, UK). Barometric pressure was recorded during each test and all gas measurements were corrected to standard room temperature and pressure for a dry gas (STPD). For each expired air sample $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated and the non-protein respiratory exchange ratio, energy expenditure and rate of energy substrate utilisation were calculated using indirect calorimetry.

2.5 Indirect calorimetry

Expired air samples were measured at rest and during exercise as described above. For each separate measurement period, mean values for $\dot{V}O_2$ and $\dot{V}CO_2$ were used in indirect calorimetry which was based on the equations from Frayn (Frayn, 1983).

No direct measure of urinary nitrogen excretion was made in any experimental chapter, thus a constant rate of nitrogen excretion of 0.00011 $g.kg^{-1}.min^{-1}$ was used, a value which has previously been applied in the literature (Flatt *et al.*, 1985;Melanson *et al.*, 2005). The constant N used in the equations below was therefore calculated as:

$$N\ (g.min^{-1}) = 0.00011 \times \text{body mass} \qquad \textbf{(Equation 2.1)}$$

Using the equations from Frayn, $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated according to the following equations:

$$\dot{V}O_2\ (l.min^{-1}) = 0.746\ CHO + 2.03\ fat + 6.04\ N \qquad \textbf{(Equation 2.2)}$$

$$\dot{V}CO_2\ (l.min^{-1}) = 0.746\ CHO + 1.43\ fat + 4.89\ N \qquad \textbf{(Equation 2.3)}$$

where CHO is carbohydrate and N is nitrogen excretion. Using equations 2.2 and 2.3, it was possible to calculate $\dot{V}O_2$ and $\dot{V}CO_2$ with a correction made for protein oxidation, thus non-protein oxygen consumption ($NP\,\dot{V}O_2$) and carbon dioxide production ($NP\,\dot{V}CO_2$) and subsequently the non-protein respiratory quotient (NPRQ) were calculated using the following:

$$NP\,\dot{V}O_2\ (l.min^{-1}) = 0.746\ CHO + 2.03\ fat - 6.04\ N \qquad \textbf{(Equation 2.4)}$$

$$NP\,\dot{V}CO_2\ (l.min^{-1}) = 0.746\ CHO + 1.43\ fat - 4.89\ N \qquad \textbf{(Equation 2.5)}$$

$$NPRQ = NP\dot{V}CO_2 / NP\dot{V}O_2 \quad \textbf{(Equation 2.6)}$$

Energy substrate oxidation, based on the protein corrected values from above, was calculated as follows:

$$\text{Fat oxidation } (g.min^{-1}) = (NP\dot{V}O_2 - NP\dot{V}CO_2) / 0.6 \quad \textbf{(Equation 2.7)}$$

$$\text{Carbohydrate oxidation } (g.min^{-1}) = NP\dot{V}O_2 - 2.03 \times \text{fat ox}) / 0.746 \quad \textbf{(Equation 2.8)}$$

$$\text{Protein oxidation } (g.min^{-1}) = N \times 6.25 \quad \textbf{(Equation 2.9)}$$

Total energy expenditure (EE) was calculated by multiplying the amount of substrate oxidised by their appropriate energy density value which were taken from Brody (Brody, 1999) and Mottram (Mottram, 1979):

$$EE\ (kJ) = (\text{fat} \times 39.0) + (\text{carbohydrate} \times 15.5) + (\text{protein} \times 17.0) \quad \textbf{(Equation 2.10)}$$

Net energy expenditure and energy substrate utilisation rates were calculated by subtracting the baseline rate from the total energy expenditure or substrate utilisation to give the rise above resting values.

2.6 Measurement of heart rate and rate of perceived exertion

Heart rate was monitored using short-range telemetry (Polar S610i Heart Rate Monitor, Polar Electro Oy, Kempele). Heart rate was monitored before, during and after all individual exercise sessions. Rate of perceived exertion (RPE) was recorded before and at regular intervals during all exercise sessions and throughout the immediate post-exercise recovery period, using the Borg Scale of 6 to 20 (Borg, 1973).

2.7 Exercise tests

The following methods describe all preliminary exercise protocols that were implemented throughout the experimental chapters. All exercise was performed in a purpose built exercise laboratory on a motorised treadmill (WOODWAY GmbH, Weil am Rhein, Germany).

2.7.1 Sub-maximal fitness tests

Prior to each fitness test, a brief familiarisation session was completed, the aim of which was to ensure subjects were familiar with how the treadmill worked, how it could be stopped in an emergency and the procedures for collecting expired air samples i.e. using

the mouthpiece and nose clip whilst walking. During this time subjects also selected a brisk walking speed they felt comfortable to maintain for extended periods of time. This self-selected speed was used in all subsequent exercise trials.

The sub-maximal fitness test was an incremental test with four stages. Prior to exercise, subjects completed a five-minute run in period with the mouthpiece and nose clip in place before a five-minute expired air collection was made and resting heart rate and RPE were recorded. Each subject started walking at their previously selected speed on a 0% gradient. Each stage of the test lasted five minutes with the gradient increasing to 3%, 6% and 9% on completion of each stage. The mouthpiece and nose clip were placed in situ three minutes into each stage and an expired air collection was taken during the final minute, during which heart rate and RPE were also recorded. If at 9% the subject had failed to reach a heart rate of at least 135 beats.minute^{-1}, an additional stage was completed with the gradient increasing to 12%.

On completion of the fitness test, the relationship between heart rate and oxygen consumption was determined for each subject. This relationship was extrapolated to the subject's maximal heart rate, predicted using 220 – age, and the corresponding maximal oxygen uptake ($\dot{V}O_2$ max) was determined. Once $\dot{V}O_2$ max had been calculated, it was possible to estimate 50% of the $\dot{V}O_2$ max, and the corresponding heart rate. Determination of the relationship between $\dot{V}O_2$ and treadmill gradient for each subject enabled the calculation of the gradient required to elicit an exercise intensity of 50% $\dot{V}O_2$ max.

2.7.2 Preliminary treadmill walk

In Chapters 3, 4 and 5, each subject returned to the exercise laboratory after their fitness test, on a separate occasion, to complete a 30-minute treadmill walk and thirty minute post-exercise recovery period. The aim of this walk was threefold. Firstly, to check the speed and gradient selected for the walk elicited an intensity of 50% $\dot{V}O_2$ max. Secondly, to calculate the energy expended during a 30-minute walk and to predict the duration of the walking intervention that would induce the target net energy expenditure, and finally to familiarise the subject with the exercise intervention and the prolonged recovery measurements that were required.

Subjects arrived at the exercise laboratory having eaten nothing for at least one hour beforehand. A resting expired air sample was collected and heart rate and RPE were

recorded. The walk commenced at the speed and gradient calculated to elicit 50% $\dot{V}O_2$ max. Three two-minute expired air collections were made at 5-7 minutes, 13-15 minutes and 28-30 minutes during the walk. Heart rate and RPE were recorded during the final minute of each gas collection. Following each collection, $\dot{V}O_2$ was calculated to check the subject was working at the correct intensity. If the workload was too high or too low, the treadmill gradient was adjusted accordingly. Once the 30-minute walk was completed, each subject immediately entered a 33-minute post-exercise recovery phase. On completion of the walk and start of recovery, the treadmill gradient was returned to 0% and subjects walked at a speed of 3.0 $km.h^{-1}$ for two minutes. After two minutes, the treadmill was stopped and the subject sat on a chair placed on the treadmill, for the remainder of the recovery phase. Throughout the 33 minutes, the mouthpiece and nose clip remained in place and continual expired air collections were made using the following timings: 0-2, 2-5, 5-8, 8-13, 13-18, 18-23, 23-28, 28-33 minutes. During the final minute of each collection, heart rate and RPE were recorded.

Once the preliminary treadmill walk was completed, the energy expended during the 30-minute walk and during the 33-minute recovery period was calculated using indirect calorimetry. The energy expended during the walk was used to estimate the duration of the walking intervention that would be required to induce the desired net energy expenditure of 27 $kJ.kg^{-1}$ body mass.

2.8 Oral fat tolerance tests and metabolic assessment

The following sections describe the methods and procedures implemented in all experimental chapters. In Chapters 3, 4 and 5 a two-day model was used; the trial intervention was completed on the afternoon prior to an oral fat tolerance test. In chapters 6 and 7 a one-day model was used; the intervention and oral fat tolerance test were completed on the same day.

2.8.1 Test meals

In Chapters 3, 4 and 5, each subject was provided with exactly the same test meal, the composition of which is shown in **Table 2.1**.

Table 2.1 Dietary composition of the test meal provided in Chapters 3, 4 and 5

	Mass (g)	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
Plain bagel	103	1095	10.9	1.7	50.7
Margarine*	24	533	0.0	14.0	0.0
Complan†	57	1052	8.8	8.5	34.8
Whole milk	250	669	8.3	9.1	11.8
Total		**3349**	**27.9**	**33.3**	**97.3**

* Polyunsaturated fat margarine, † Complan formula in powder form and strawberry flavour provided by Complan Foods Ltd, Windsor, UK.

The test meal was provided on completion of all fasting measurements and exactly the same meal was given again, 4.5 hours later. All subjects were advised to consume each test meal within 10 minutes and water was not allowed during this time. In total, 48% of energy was provided as carbohydrate, 38% as fat and the remaining 14% as protein. The plain bagel was lightly toasted, with the margarine spread by the experimenter before being given to the subject. The Complan formula was thoroughly mixed with whole milk to form a milkshake type drink.

In Chapters 6 and 7, a single test meal was provided either following a 60-minute treadmill walk in the high energy turnover trial or an equivalent period of rest in the low energy turnover trial. Again, subjects were advised to consume the meal within 10 minutes and water was not allowed during this time. In the low energy turnover trial, the test meal comprised exactly the same foods as those used above in Chapters 3, 4 and 5, except that there was a slight reduction in margarine and increase in milk content to improve the palatability of the meal. The energy content of the meal was individually calculated for each subject to maintain energy balance over the six-hour observation period as described in section 2.4.1.1. Exactly the same meal was provided in the high energy turnover trial except that the energy content of the meal was increased to replace 110% of the net energy expended during the treadmill walk. To determine the amount of food needed in the test meal, firstly, the quantity of food required to provide 3356 kJ of energy was calculated (**Table 2.2**). These quantities were then multiplied by a conversion factor to determine the amount of food needed to provide the specific energy requirements for each subject i.e. to provide 2510 kJ, the weight of each food was multiplied by 0.75 (2510 divided by 3356). The foods were prepared in exactly the same way as above i.e. the bagel was lightly toasted with the margarine pre-spread by the experimenter and the milk and Complan

formula were mixed to form a milkshake drink. In total, 49% of energy was provided as carbohydrate, 37% as fat and the remaining 14% as protein.

Table 2.2 The sample meal from which the composition of the test meal provided in Chapters 6 and 7 was calculated.

	Mass (g)	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
Plain bagel	104	1110	9.5	1.8	52.4
Margarine*	20	444	0.0	11.7	0.0
Complan†	57	1052	8.8	8.5	34.8
Whole milk	280	750	9.2	10.2	13.2
Total		**3356**	**27.5**	**32.1**	**100.4**

* Polyunsaturated fat margarine, † Complan formula in powder form and strawberry flavour provided by Complan Foods Ltd, Windsor, UK.

2.8.2 Metabolic assessment

2.8.2.1 Blood sampling

In Chapters 3, 4 and 5, regular venous blood samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 5.5, 6.5, 7.5 and 8.5 hours during the postprandial metabolic assessment. In Chapters 6 and 7, regular venous blood samples were taken at -2 (high energy turnover trial only), 0, 0.5, 1, 2, 3, 4, 5 and 6 hours during the postprandial metabolic assessment. All blood samples were collected via a cannula (Becton Dickinson Infusion Therapy AB, Helsinborg, Sweden) placed within an antecubital vein. If a blood sample could not be successfully collected via the cannula, a blood sample was collected by venopuncture of an antecubital vein instead. Once the cannula was situated within the vein, a three-way valve (Becton Dickinson Infusion Therapy AB, Helsinborg, Sweden) was attached to control blood flow. A 10-minute settling period was completed after the cannula was introduced and before a baseline blood sample was collected. Blood samples were collected into ethylenediamine tetra-acetic acid (EDTA) containing tubes (BD Vacutainer Systems, Plymouth, UK) via a vacutainer and adaptor (BD, Franklin Lakes, NJ, USA) which connected the cannula valve directly to the EDTA tube, and avoided the use of syringes for blood collection. Throughout all metabolic assessments, blood samples were collected with the subject in a supine position and the cannula was kept patent by regularly flushing with a 0.9% sodium chloride saline solution (B.Braun, Melsungen, Germany). On collection into the EDTA tubes, all samples were immediately placed onto ice before being centrifuged and frozen at -80°C for analysis.

2.8.2.2 Pulse Wave Velocity

Prior to all measurements of pulse wave velocity (PWV), subjects lay supine for at least 10 minutes. All measurements recorded in Chapters 4 and 6 were made using the Complior (Artech Medical, Pantin, France), a valid and reliable automated device (Asmar *et al.*, 1995), on the right hand side of the body, with the subject lying relaxed on the couch.

In Chapter 4, PWV was measured between the carotid and radial sites. The subject's physiological data including their weight, height, blood pressure and waist and hip circumferences were entered into the Complior program prior to any measurement. Once the test started, a sensor was positioned over the radial artery and held in place using a clamp device. The position of the sensor was adjusted until a clear pulse signal was observed on the computer screen. A second sensor situated above the carotid artery was held in place by the experimenter, again ensuring a clear pulse signal was visible. The positioning of the sensors can be seen in **Figure 2.1. Figure 2.2** shows an example of the pulse signals as they appear on the computer screen. Once a good signal was obtained and had persisted for approximately 10 seconds, the foot pedal was pressed to halt the signal. The distance between the two sensors was measured using a non-elastic tape measure, over the surface of the skin, following the natural direction of the artery i.e. from the carotid pulse site, along the line of the clavicle and towards the shoulder joint, then down to the radial pulse site. The carotid-radial PWV was automatically calculated using the following equation:

$$\text{PWV } (m.s^{-1}) = \text{arterial distance (m)} / \text{transit time (s)} \qquad \textbf{(Equation 2.11)}$$

Six PWV measurements were made at each time point with their mean used in further analysis. In Chapter 4, carotid-radial PWV was measured at zero hours and again at 2, 4, 6.5 and 8.5 hours.

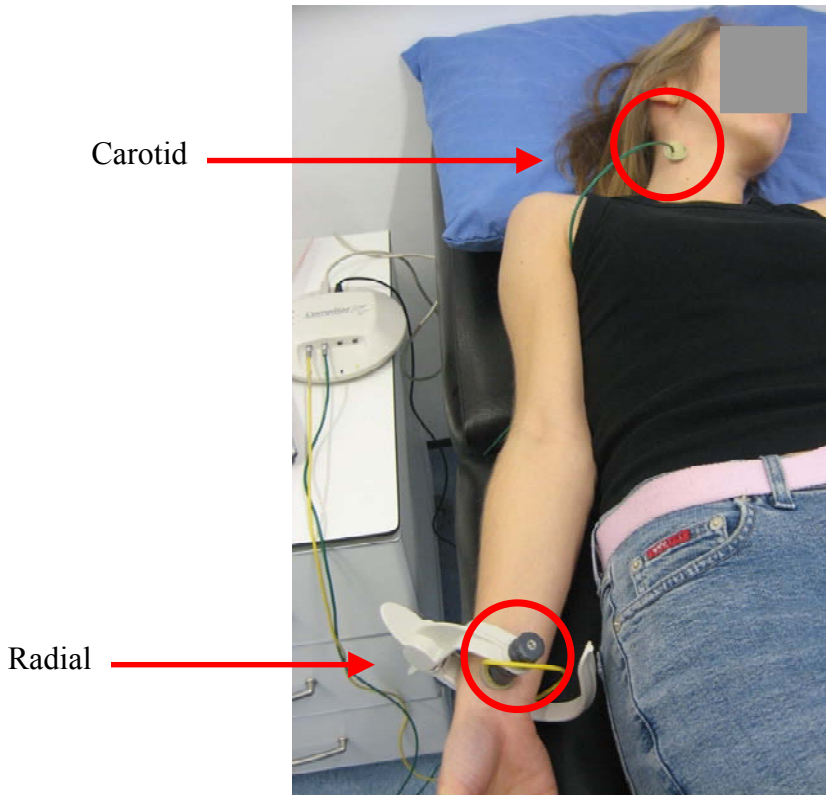


Figure 2.1. Locations of the carotid and radial pulse sites between which peripheral PWV was measured.

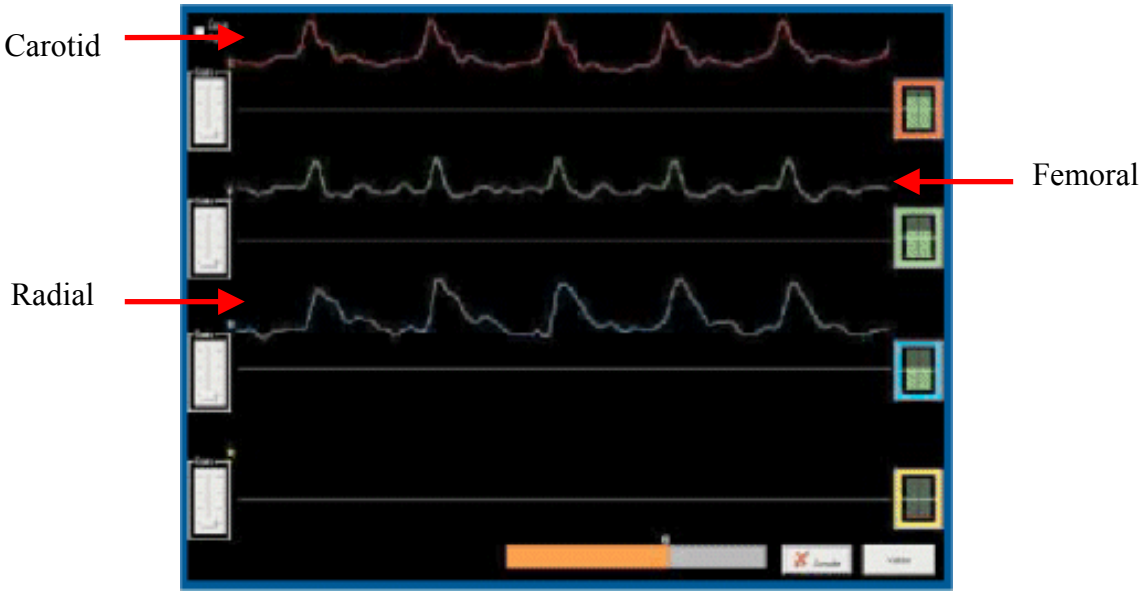


Figure 2.2 An example of the carotid, femoral and radial pulse signals observed during the measurement of PWV.

In Chapter 6, carotid-radial PWV was measured in exactly the same was as described above for Chapter 4 and as shown in Figure 2.1. Further PWV measurements were made between the carotid and femoral pulse sites. The femoral site was located by the subject themselves following verbal instructions from the experimenter. The sensor was positioned above the femoral pulse and adjusted until a clear pulse signal was received. Throughout the measurement period, the subject held the femoral sensor in position whilst the second sensor was situated above the carotid pulse and held securely by the experimenter. The positioning of these sensors can be seen in **Figure 2.3**. Once a clear signal had persisted for at least 10 seconds, the foot pedal was pressed and the signal suspended. The distance between the sensors was again measured using a non-elastic tape measure; the distance used was that between the suprasternal notch and the femoral sensor. Carotid-femoral PWV was automatically calculated using Equation 2.11. In Chapter 6, PWV was measured at both sites at -2 (high energy turnover trial only), 0, 3 and 6 hours and at each time point, three carotid-radial and three carotid-femoral PWV measurements were recorded with the average used in further analysis.

Figure 2.3 Locations of the carotid and femoral pulse sites between which central PWV was measured.

2.8.3 Subjective ratings of appetite and food palatability

Subjective ratings of appetite were recorded at 0, 0.5, 1, 2, 3, 4, 5, 5.5, 6.5, 7.5 and 8.5 hours in Chapter 5. In Chapter 7, subjective ratings of appetite were measured at -2 (high energy turnover trial only), 0, 0.5, 1, 2, 3, 4, 5 and 6 hours. Subjective ratings of food palatability were also measured in Chapter 7 during the test meal and the buffet meal. Appetite and food palatability were measured using 10 cm VAS questionnaires based on those described by Flint *et al*. (Flint *et al.*, 2000) For both types of questionnaire, subjects

were asked to read each question before making a clear slash through the solid line at the position which most closely represented their current state. On its completion, each questionnaire was collected by the experimenter so that the subject could not refer back to it at later time points. The questionnaires were analysed by the experimenter by measuring the distance (in mm) from the left hand side of the line to the point at which the slash crossed through it.

An example of the appetite VAS questionnaire is given in **Appendix 2A.** A series of nine questions were completed. Each question was followed by a 10 cm solid line which had statements anchored at each end, each statement representing the extreme and opposite answers to the question, for example the question 'How hungry do you feel?' included the statements 'I am not hungry at all' and 'I have never been more hungry'. The nine questions that were included in the questionnaire were:

1. How hungry do you feel (now)?
2. How satisfied do you feel (now)?
3. How full do you feel (now)?
4. How much do you think you can eat (now)?
5. How strong is your desire to eat (now)?
6. Would you like to eat something sweet (now)?
7. Would you like to eat something salty (now)?
8. Would you like to eat something savoury (now)?
9. Would you like to eat something fatty (now)?

An example of the food palatability questionnaire is given in **Appendix 2B.** A series of five questions were completed and similar to the appetite questionnaire, each question was followed by a 10 cm line anchored at each end with extreme and opposite statements, for example the question 'How appealing does your food look?' was coupled with the statements 'Good' and 'Bad' at the ends of the line. The five questions that were included in the palatability questionnaire were:

1. How appealing does your meal look?
2. How does your meal smell?
3. How does your meal taste?
4. How much of an aftertaste has your meal left?
5. How palatable have you found the meal?

2.9 Pre-experimental trial controls

Each subject was provided with a set of guidelines which they were instructed to follow during the three days preceding each experimental trial. In Chapters 3, 4 and 5, these three days were those immediately prior to day two of the experimental protocol. In Chapters 6 and 7, these three days were those leading up to each of the one day experimental trials. In all experimental chapters, subjects were asked to avoid drinking any alcohol during these days. They were also asked to avoid all planned exercise with the exception of the two separate walking interventions that were completed in Chapters 3, 4 and 5.

In Chapters 3, 4 and 5 subjects were provided with all of their food and drink during the three-day control period. The aim of this diet was to ensure subjects remained in energy balance leading up to each of the three trials. The energy content of the food and drinks provided on each day was based on previous calculations of the subjects' daily energy expenditure as described above in section 2.4.1.1. Prior to the provision of each three-day diet, subjects were asked to complete a questionnaire giving information on their i) preferred breakfast cereal, ii) preferred milk, iii) preferred type of bread, iv) choice of sandwich filling, v) crisp flavour preference, vi) tea and coffee consumption and vii) food dislikes. The full questionnaire can be seen in **Appendix 3A.** The meals were designed to provide 20% of the daily energy intake at breakfast, 35% at lunch and 45% at dinner. Overall, the macronutrient composition of the food was designed to match the typical Scottish daily intakes and as such provided 49% of energy as carbohydrate, 37% as fat, and the remaining 14% as protein (Department for Environment Food and Rural Affairs, 2004). An example of the three day diet can be seen in **Table 2.3.**

Table 2.3 An example of foods provided during the three days preceding each experimental trial.

	Day 1	Day 2	Day 3
Breakfast	Crunchy nut cornflakes	Crunchy nut cornflakes	Crunchy nut cornflakes
	Wholemeal bread	Wholemeal bread	Wholemeal bread
	Margarine*	Margarine*	Margarine*
	Semi-skimmed milk	Semi-skimmed milk	Semi-skimmed milk
Lunch	Wholemeal bread	Wholemeal rolls	Wholemeal bread
	Margarine*	Margarine*	Margarine*
	Cheddar cheese	Cheddar cheese	Cheddar cheese
	Ham	Ham	Ham
	Ready salted crisps	Ready salted crisps	Ready salted crisps
	Strawberry yoghurt	Twix bar	Dairy milk bar
Dinner	Chicken risotto	Macaroni cheese	Cheese & Ham pasta
	Wholemeal bread	Wholemeal bread	Pasta sauce
	Margarine*	Margarine*	Wholemeal bread
	Chocolate mousse	Fruit cocktail†	Margarine*
			Strawberry yoghurt
Drink	Orange squash	Orange squash	Orange squash
Snacks	1 x Apple	1 x Apple	1 x Apple
	1 x packet crisps	1 x packet crisps	1 x packet crisps

***** Polyunsaturated fat margarine, † fruit cocktail was served in juice

Prior to being given to the subject, each of the foods included in the diet were individually weighed (A & D Instruments, Oxford, UK), packaged and labelled. The type and variety of foods that were used can be seen in **Figure 2.4** both before and after preparation. In addition to the main meals, each subject was provided with two optional snacks for each of the three days, an apple and a packet of crisps, which they were allowed to consume only if they suffered extreme hunger. They were also provided with a pre-weighed bottle of sugar free, concentrated orange squash which they were able to consume freely. During the three day diet, all subjects completed a 'checklist' to ensure all foods and the correct foods for that day were eaten. On completion of the diet all food tubs and packaging were returned to the experimenter. If subjects had not consumed all of the food or squash provided or if they had eaten any of the snacks during the three days leading up to the first experimental trial, this information was recorded and the diet was adjusted accordingly before being provided again for the days preceding subsequent trials. All subjects were strictly

instructed not to consume any other food or drink other than those provided by the experimenter and compliance was monitored daily via telephone and or email contact.

Figure 2.4 Photographs of the foods provided in the three days preceding experimental trials before (top) and after (bottom) preparation.

In Chapters 6 and 7, for the three days preceding the main experimental trial, each subject was asked to weigh and record all of the food and drink they consumed during those days; they were provided with a record sheet (**Appendix 3B**) and weighing scales (Salter Housewares Ltd, Kent, UK) to help them do this. A full set of written instructions were provided with the data collection sheet and a verbal explanation was also offered by the experimenter. Throughout the three days, subjects were instructed to contact the experimenter if they were unsure or had any questions about how to record foods accurately. On completion of the first trial, subjects were provided with a copy of their

three-day food and drink intakes which they were instructed to copy exactly for the three days preceding the subsequent experimental trial.

2.10 Blood analysis

In all experimental chapters, blood samples were collected into EDTA tubes and immediately placed on ice. Prior to centrifugation, 1.8 ml of blood was extracted in preparation for total ghrelin analysis (Chapters 5 and 7) and a further 1.98 ml of blood was extracted in preparation for acylated ghrelin analysis (Chapter 7).

In all experimental chapters, blood samples were removed from ice and placed into a refrigerated centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Samples were spun at 3000 revolutions per minute (rpm) for 15 minutes, at 4°C. Once the plasma and red blood cells were separated, 0.5 ml aliquots of plasma were extracted and placed into 2 ml labelled eppendorfs (Alphalaboratories, Hampshire, UK). All samples were frozen at -80°C until analysis. In Chapters 5 and 7, to prevent the degradation of the hormone of interest, blood samples in which total ghrelin and acylated ghrelin were to be measured were treated separately. As reported above, 1.8 ml of blood was extracted in preparation for total ghrelin analysis. This 1.8 ml of blood was added into a 2 ml eppendorf containing 144 µl of bovine aprotinin (Sigma-Aldrich Company Ltd, Dorset, England). The eppendorf was inverted gently 10 times to mix the blood and aprotinin before being centrifuged (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) for five minutes at 14000 rpm. Two separate 0.5 ml aliquots of the treated plasma were removed and added into labelled 2 ml eppendorfs before being stored at -80°C. In Chapter 7, blood samples in which acylated ghrelin were to be measured were also treated separately. 1.98 ml of blood was extracted and added into a 2 ml eppendorf containing 20 µl of p-hydroxymercuribenzoic acid (Sigma-Aldrich Company Ltd, Dorset, England). The eppendorf was inverted gently 10 times and then centrifuged for 5 minutes at 14000 rpm. Once separated, 1.0 ml of the treated plasma was extracted and added to a 2 ml eppendorf containing 100 µl of 1N hydrochloric acid solution (Sigma-Aldrich Company Ltd, Dorset, England). Again the eppendorf was gently inverted 10 times before being centrifuged for a further 5 minutes at 14000 rpm. Two 0.5 ml aliquots of the treated plasma were extracted, added into labelled eppendorfs and frozen at -80°C.

2.10.1 Enzyme-linked immunoassay (ELISA) procedures

Full details of the insulin, total ghrelin, acylated ghrelin and ADMA ELISAs are given in **Appendices 4A, 4B, 4C** and **4D**, respectively. All ELISA procedures were based on a

'sandwich' technique. The wells of the plates were coated with antibody specific to the protein of interest being measured in plasma. When plasma was added to the wells, the protein bound with the antibody with any unbound molecules being removed by a washing process. A second antibody was added which was specific for a separate site on the protein and again, washing removed any unbound particles. Attached to this second antibody was an enzyme, the activity of which produced a coloured solution, the intensity of which was proportional to the amount of protein present in the plasma sample (Stryer, 1988).

Insulin analysis was performed in the Vascular Biochemistry Department at Glasgow Royal Infirmary or in the biochemistry laboratory at Yorkhill Children's Hospital using an ELISA with < 0.01% cross-reactivity with pro-insulin (Mercodia AB, Uppsala, Sweden). Total ghrelin was also analysed at Yorkhill Children's Hospital (Phoenix Europe GmbH, Karlsruhe, Germany), whereas acylated ghrelin was analysed in the biochemistry laboratory at the University of Glasgow (SPI-BIO, Montigny Le Bretonneux, France). A further ELISA was completed for the analysis of ADMA and this was performed at Glasgow Royal Infirmary (DLD Diagnostika GmbH, Hamburg, Germany). All samples were analysed in duplicate and all ELISA procedures were completed by the author.

2.10.2 Spectrophometric procedures

Plasma TG (**Appendix 4E**), NEFA (**Appendix 4F**), glucose (**Appendix 4G**), total cholesterol (**Appendix 4H**) and HDL cholesterol concentrations (**Appendix 4I**) (Roche Diagnostics GmbH, Mannheim, Germany. Wako Chemicals GmbH, Germany) were determined at Glasgow Royal Infirmary. LDL concentrations were calculated using the Friedwald equation (Friedewald *et al.*, 1972). 3-hydroxybutyrate concentrations (**Appendix 4J**) were measured at the University of Glasgow (Randox Laboratories Ltd, Co. Antrim, Ireland). All measurements for TG, NEFA, glucose and cholesterol were performed on an ILAB^{TM} 600 (Instrumentation Laboratory, USA). 3-hydroxybutyrate was measured using a Cobas Mira Plus (ABX Diagnostics, France). All analyses were performed on fully defrosted plasma samples. Plasma TG, NEFA, glucose, total cholesterol, HDL cholesterol and LDL cholesterol were all determined by colleagues at Glasgow Royal Infirmary. Plasma 3-OHB analyses were completed by the author.

2.10.3 Radioimmunoassay (RIA) procedures

Leptin was analysed by colleagues in the Clinical Biochemistry Department at Glasgow Royal Infirmary using an RIA method developed internally (McConway *et al.*, 2000). Further details of the analysis are given in **Appendix 4K**.

2.10.4 Accuracy and precision of blood analysis procedures

Quality control sera were used to monitor the accuracy and precision of the different analysis (Mercodia AB, Uppsala, Sweden. Phoenix Europe GmbH, Karlsruhe, Germany. SPI-BIO, Montigny Le Bretonneux, France. R & D Systems Europe, Oxford, UK. DLD Diagnostika GmbH, Hamburg, Germany. Roche Diagnostics GmbH, Mannheim, Germany. Randox Laboratories Ltd, Co. Antrim, Ireland. Wako Chemicals GmbH, Germany). For all analyses, samples for individual subjects were run on a single analyser run, or a single ELISA plate. Within-batch coefficients of variation were < 4.0% for all non-ELISA assays. The within-plate coefficients of variation for ELISA assays were < 4% for insulin, < 10% for total ghrelin, < 6% for acylated ghrelin and < 10% for ADMA.

CHAPTER 3
ENERGY REPLACEMENT ATTENUATES THE EFFECTS OF PRIOR MODERATE EXERCISE ON POSTPRANDIAL METABOLISM IN OVERWEIGHT/OBESE MEN

3.1 Introduction

Metabolic perturbations occurring during the postprandial period are implicated in the development and progression of atherosclerosis by a number of plausible mechanisms. Postprandial lipoproteins and their remnants may deposit into arterial walls accelerating the development of atheromous plaques (Zilversmit, 1979) and the postprandial elevation in circulating TG-rich lipoproteins contributes to the accumulation of small, dense LDL and a decrease in cardio protective HDL; a combination known as the atherogenic phenotype (Cohn, 1998). In addition, postprandial hyperinsulinemia, together with the transient postprandial increase in insulin resistance may also contribute to atherogenic progression (Boquist *et al.*, 2000;Frayn, 2002b;Tsuchihashi *et al.*, 1999) and the development of chronic insulin resistance and type II diabetes (Yki-Jarvinen, 1990). As humans spend the majority of the day in the postprandial state, interventions which alter postprandial metabolism may have implications for the prevention and management of metabolic diseases.

It is well established that moderate exercise influences a number of aspects of postprandial metabolism. Postprandial TG and insulin concentrations are lower on the day following exercise (Gill *et al.*, 2002b): these changes contribute to an improved cardio-metabolic risk profile following exercise. In addition, an increase in postprandial fat oxidation is evident for at least 24 hours following an exercise session (Hansen *et al.*, 2005).

The effects of exercise on postprandial metabolism, at least with respect to the attenuation of TG concentrations, are related to the energy expended during the exercise session, independent of the duration or intensity of exercise *per se* (Tsetsonis, 1996). However, it has previously been shown that energy deficit is not the sole determinant of the exercise-induced changes in postprandial metabolism, as a dietary-induced energy deficit induces a smaller attenuation in postprandial TG concentrations than an equivalent exercise-induced deficit (Gill & Hardman, 2000). It remains unclear whether favourable effects of exercise on postprandial metabolism are still evident when there is an increase in energy intake to compensate for energy deficits caused by exercise.

The purpose of the present study was therefore to determine the extent to which exercise-induced changes to postprandial metabolism, particularly with respect to lipid metabolism, insulin/glucose dynamics and energy substrate utilisation, would occur in the absence of an exercise-induced energy deficit. This is of clinical, as well as mechanistic, importance as adult individuals often increase energy intake when they exercise (Pomerleau *et al.*, 2004;Stubbs *et al.*, 2002b), thus compensating for the extra energy deficit caused by exercise. Overweight and obese middle-aged men, with an increased waist circumference – a group at increased risk of cardio-metabolic disease, who are typically targeted with exercise for health interventions – were chosen for study.

3.2 Methods

3.2.1 Subjects

Thirteen men were recruited to this study. Their physical characteristics were age: 40 ± 8 years, body mass index (BMI): 31.1 ± 3.0 $kg.m^{-2}$, waist circumference: 105.6 ± 6.6 cm, systolic blood pressure (BP): 132 ± 11 mmHg, diastolic BP: 81 ± 4 mmHg and maximal oxygen uptake ($\dot{V}O_2$max): 39.3 ± 5.7 $ml.kg^{-1}.min^{-1}$ (mean ± SD). All subjects were apparently healthy, non-diabetic, non-smokers with no known cardiovascular contraindications to exercise as assessed by a Bruce protocol exercise ECG test (Chapter 2.2). None was taking any medication thought to interfere with lipid or carbohydrate metabolism. One subject was taking Analaprol for hypertension and this was continued at a stable dosage throughout the study. On medication his blood pressure at screening was 135/82 mmHg.

3.2.2 Experimental design

After preliminary testing, each subject completed three two-day trials in random order, separated by an interval of at least one week. Day-one involved one of three interventions: control, exercise with energy deficit (energy-deficit) or exercise with energy replacement (energy-replacement). Day-two comprised an 8.5-hour metabolic assessment, described in detail below, and took exactly the same format for each trial. An overview of the two-day protocol is shown in **Figure 3.1**.

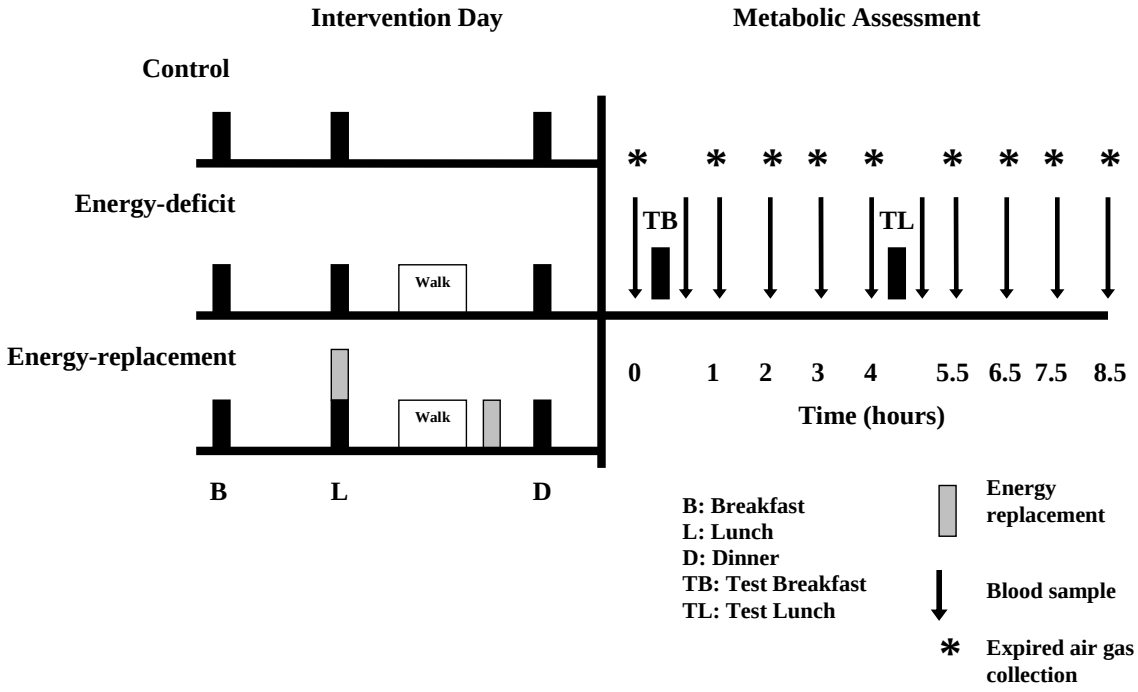


Figure 3.1 An overview of the two-day study design

3.2.3 Preliminary exercise test

A four-stage preliminary sub-maximal treadmill test (Chapter 2.7.1) was completed prior to the first intervention day in order to estimate $\dot{V}O_2$ max and calculate the speed and gradient required to elicit the intensity of 50% $\dot{V}O_2$ max (the intensity used for the exercise interventions). A 30-minute treadmill walk, described in Chapter 2.7.2, was completed on a separate occasion to confirm the speed and gradient calculated to elicit this required intensity was correct.

3.2.4 Estimation of daily energy requirements

Prior to the first trial, subjects visited the metabolic suite following a 12-hour overnight fast for measurement of their resting metabolic rate an overview of which is given in Chapter 2.4.1. Briefly, after 20 minutes of rest during which subjects lay supine, expired air measurements were collected over 25 minutes from which metabolic rate and substrate utilisation were determined by indirect calorimetry (Chapter 2.5) using a ventilated hood. Daily energy requirements were calculated on an individual basis using a physical activity level of 1.55 (Chapter 2.4.1.1).

3.2.5 Day-one: Experimental trial interventions

In both exercise trials below, exercise was completed in the afternoon of day one of the two-day trials, starting approximately two hours after lunch. During all exercise

interventions subjects were allowed to listen to music on the stereo or a personal music player and they were provided with water ad libitum throughout.

3.2.5.1 Control trial

For two days prior to and on the intervention day of the control trial (i.e. Day-one), subjects refrained from alcohol and all planned exercise. During this three-day period, subjects were provided with all of their food and drink by the experimenters, in diets designed to maintain energy balance (Chapter 2.9). Macronutrient composition of the meals reflected the average Scottish diet (37% of energy from fat, 49% carbohydrate and 14% protein) and daily energy intake was 12.5 ± 1.4 MJ.

On the intervention day of the control trial, subjects were specifically instructed to sit or lie quietly for a two-hour period in the afternoon, starting approximately two hours after lunch, which corresponded to the time of exercise in the other two trials.

3.2.5.2 Exercise with energy deficit trial

After a five minute run in, two separate five minute resting expired air collections were made. A mean of the gas composition measurements for each bag was used to determine resting, baseline values for $\dot{V}O_2$ and $\dot{V}CO_2$. Heart rate and RPE (Chapter 2.6) were measured during the final minute of the second gas collection. The treadmill walk started at the speed and gradient required to elicit an intensity of 50% $\dot{V}O_2$ max. Expired air samples were collected during the two minutes preceding each 15-minute time point i.e. 13-15, 28-30, 43-45 minutes etc. Heart rate and RPE were recorded during the final minute of each measurement. Following each gas collection, $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated to monitor the exercise intensity. The net energy expended during each 15-minute stage was calculated using indirect calorimetry. Exercise was terminated once subjects had walked for sufficient time to expend 27 $kJ.kg^{-1}$ body mass and subsequently the duration of the treadmill walk varied from 75 to 120 minutes. An expired air collection was made during the final two minutes of exercise, before entering the recovery period. Post-exercise recovery measurements were made for the 33 minutes following the end of exercise in exactly the same way as described above for the preliminary treadmill walk. On completion of the walk, subjects were allowed to shower and return home or back to work.

3.2.5.3 Exercise with energy replacement trial

Exactly the same exercise protocol was completed as described above for the exercise with energy-deficit trial, however the net energy expended during the treadmill walk was replaced to eliminate any exercise-induced energy deficit. For each subject, their target net energy expenditure, and thus the amount of energy to be replaced, was predicted as 27 kJ multiplied by their body mass i.e. 2700 kJ for a 100 kg subject. It has been suggested that metabolic rate can remain elevated for some time after exercise and the energy expenditure associated with such an increase in metabolic rate has been suggested to range from 2-7% of the energy expend during exercise (Gore & Withers, 1990;Laforgia *et al.*, 1997;Sedlock *et al.*, 1989). Therefore, to ensure the entire exercise-induced energy deficit was eliminated, the predicted energy replacement was increased by 10% and in total provided 110% of the net energy expended during exercise. Energy was replaced using a meal replacement drink (Complan, Complan Foods Ltd, Windsor, UK) made up with whole milk and in total provided 48% of energy as carbohydrate, 38% as fat and the remaining 14% as protein. Half (55%) of the predicted energy replacement was provided with the subjects' lunch. On completion of the exercise intervention, the remainder of the energy to be replaced was calculated using indirect calorimetry, based on the actual net energy expenditure of the walk, subtracting the 55% of energy already consumed at lunch. The remaining energy was replaced on completion of the post-exercise recovery period, approximately 35 minutes after exercise was completed.

3.2.6 Day-two: Metabolic assessment

Subjects reported to the metabolic suite after an overnight fast of at least twelve hours, approximately sixteen hours after completion of exercise in the energy-deficit and energy-replacement trials. Following a 10-minute rest lying on a couch, a 30-minute expired air sample was collected (Chapter 2.4.1). A cannula was introduced into an antecubital vein, and a fasting blood sample was taken as described in Chapter 2.8.2.1.

Once fasting measurements were made, the test breakfast, described in Chapter 2.8.1 was provided. Briefly, this comprised a bagel, polyunsaturated fat margarine and a meal replacement drink (Complan, Complan Foods Ltd, Windsor, UK) made with whole milk, and provided 3.34 MJ of energy, 97g CHO, 33g fat and 28g protein. Exactly the same meal was given again at lunch, 4.5-hours later.

During the 8.5-hour observation period, blood samples were collected at 0.5, 1, 2, 3 and 4 hours after breakfast and the pattern was repeated after lunch (5, 5.5, 6.5, 7.5 and 8.5 hours

post breakfast). Expired air samples (Chapter 2.4.1) were taken immediately preceding the 1, 2, 3, 4, 5.5, 6.5, 7.5 and 8.5-hour time points (Figure 3.1). Water was provided ad-libitum during the first trial with the pattern and amount of water ingested repeated in subsequent trials.

3.2.7 Blood analysis

In each trial, total, HDL and LDL cholesterol, TG, NEFA, glucose, insulin and 3-OHB concentrations were determined in the fasting state. Plasma TG, NEFA, glucose, insulin and 3-OHB were also determined at regular intervals throughout the postprandial observation period. A more detailed description of all these analyses is given in Chapter 2.10.

3.2.8 Statistical analysis

Data were analysed using Statistica (version 6.0, StatSoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennsylvania). Prior to analysis, all data were tested for normality using the Anderson-Darling normality test and, if necessary, logarithmically transformed. Subsequently, plasma TG concentrations were transformed prior to any analyses.

Insulin sensitivity was estimated using the homeostasis model assessment (HOMA) (Matthews *et al.*, 1985). Energy expenditure and substrate utilisation during the trial walks, and on metabolic assessment days, were calculated using indirect calorimetry and for these calculations, urinary nitrogen excretion was assumed to be 0.11 $mg.kg^{-1}.min^{-1}$ throughout all trials (Chapter 2.5). The total area under the 8.5-hour variable *vs*. time curves (AUC), calculated using the trapezium rule, and the incremental AUC, calculated as the increment in AUC over baseline concentrations, were used as summary measures of the postprandial responses. It should be noted that, for variables where values decrease from baseline postprandially, the calculated incremental AUC is negative and represents the area above the curve extending up to the baseline value, providing an index of postprandial suppression. Differences between the three trials for fasting values and summary postprandial responses were analysed using repeated measures one-way analysis of variance (ANOVA). Differences over time for the three trials were calculated using two-way ANOVA with repeated measures for trial and time. Post hoc Fisher least significant difference tests were used to identify where differences lay when main trial and or interaction effects were observed. Relationships between variables were assessed using Pearson product-moment correlations. Multiple regression analyses were performed to

establish the independence of relationships. Statistical significance was accepted at the $p < 0.05$ level and data are presented as mean ± SEM, unless otherwise stated.

3.3 Results

3.3.1 Responses during treadmill walk

For each subject, the walk durations in the energy-deficit and energy-replacement trials were identical. In both trials subjects walked for 90.8 ± 3.6 minutes at a speed of 5.5 ± 0.1 $km.h^{-1}$ up a gradient of 3.4 ± 0.5%. Net energy expenditure of the walk was 2.82 ± 0.12 MJ and 2.81 ± 0.12 MJ for energy-deficit and energy-replacement, respectively. Net carbohydrate oxidation during the trial walks was 108.8 ± 7.6 g for energy-deficit and 114.5 ± 7.6 g for energy-replacement and net fat oxidation was 24.4 ± 2.7 g for energy-deficit and 21.7 ± 1.8 g for energy-replacement. Mean $\dot{V}O_2$ and heart rate during the walks was 19.1 ± 0.6 $ml.kg^{-1}.min^{-1}$ and 122 ± 3 $beat.min^{-1}$, respectively, for energy-deficit and 19.3 ± 0.6 $ml.kg^{-1}.min^{-1}$ and 123 ± 3 $beat.min^{-1}$, respectively, for energy-replacement. For both walks, subjects rated the exercise intensity as "fairly light" (energy-deficit: 10.8 ± 0.5, energy-replacement: 10.4 ± 0.6) on the Borg scale of 6-20 (Borg, 1973). There were no significant differences between exercise trials in any of these variables.

3.3.2 Day-Two: Metabolic assessment

3.3.2.1 Responses in the fasted state

A summary of all fasting concentrations is shown in **Table 3.1**. Fasting TG concentrations were 16% lower in energy-deficit compared with control ($p < 0.01$). Fasting 3-OHB concentrations were 111% higher in energy-deficit compared with control ($p < 0.05$). There were no significant differences between trials in fasting NEFA, LDL or HDL cholesterol or fasting glucose and insulin concentrations. There were also no significant differences between trials in HOMA-estimated insulin resistance.

Data for metabolic rate and energy substrate utilisation in the fasted state are also reported in Table 3.1. The rate of fat oxidation was higher in energy-deficit than energy-replacement and control by 19% ($p < 0.05$) and 32% ($p < 0.01$), respectively. Reciprocally, carbohydrate oxidation was 28% lower in energy-deficit compared with control ($p < 0.05$).

Table 3.1. Fasting plasma and metabolic values

	Control	Energy-deficit	Energy-replacement
Triglyceride ($mmol.l^{-1}$)	1.34 ± 0.19	1.12 ± 0.17[a]	1.21 ± 0.13
NEFA ($mmol.l^{-1}$)	0.35 ± 0.02	0.41 ± 0.02	0.41 ± 0.03
Glucose ($mmol.l^{-1}$)	5.28 ± 0.13	5.19 ± 0.12	5.17 ± 0.14
Insulin ($\mu U.l^{-1}$)	9.45 ± 1.08	9.05 ± 1.17	8.85 ± 1.20
HOMA-IR	2.25 ± 1.10	2.10 ± 1.03	2.05 ± 1.10
3-hydroxybutyrate ($\mu mol.l^{-1}$)	45 ± 4	95 ± 3[a]	53 ± 7
Cholesterol ($mmol.l^{-1}$)	4.54 ± 0.23	4.41 ± 0.23	4.39 ± 0.26
HDL ($mmol.l^{-1}$)	1.09 ± 0.09	1.05 ± 0.07	1.10 ± 0.08
LDL ($mmol.l^{-1}$)	2.85 ± 0.19	2.83 ± 0.23	2.84 ± 0.21
Respiratory Exchange Ratio	0.84 ± 0.01	0.81 ± 0.01[a,b]	0.84 ± 0.01
Energy Expenditure ($kJ.hr^{-1}$)	326.9 ± 11.0	330.6 ± 9.6	325.7 ± 10.3
Fat Oxidation ($g.hr^{-1}$)	3.24 ± 0.29	4.28 ± 0.34[a,b]	3.47 ± 0.42
Carbohydrate Oxidation ($g.hr^{-1}$)	7.61 ± 0.75	5.49 ± 0.76[a]	7.03 ± 1.04

N =13, values are mean ± SEM. [a]different from control ($p < 0.05$), [b]different from energy-replacement ($p < 0.05$). NEFA: non-esterified fatty acids, HOMA-IR: homeostasis model assessment of insulin resistance, HDL: high-density lipoprotein, LDL: low-density lipoprotein.

3.3.2.2 Postprandial responses

The postprandial responses for TG and for NEFA, 3-OHB, insulin and glucose, are shown in **Figure 3.2** and **Figure 3.3**, respectively. Summary measures of these responses are given in **Table 3.2**. The postprandial TG total AUC in energy-deficit was 14% lower than control ($p < 0.05$) and 10% lower than energy-replacement ($p < 0.05$). Postprandial NEFA total AUC was 14% higher than control in both exercise trials ($p < 0.01$ for both). The postprandial 3-OHB total AUC in energy-deficit was 40% higher than control ($p < 0.001$) and 19% higher than energy-replacement ($p < 0.05$). Compared with control, the postprandial insulin total AUC was 18% lower in energy-deficit ($p < 0.001$) and 10% lower in energy-replacement ($p < 0.01$). The postprandial insulin total AUC was 10% lower in energy-deficit than energy-replacement ($p < 0.05$). There were no significant differences in postprandial glucose total AUC between the three trials.

Table 3.2. Summary postprandial responses

	Control	Energy-deficit	Energy-replacement
Triglyceride (mmol.l^{-1}.h)			
Total AUC	17.09 ± 2.38	14.71 ± 2.21[a,b]	16.15 ± 1.79
Incremental AUC	5.70 ± 0.94	5.10 ± 0.85	5.87 ± 0.77
NEFA (mmol.l^{-1}.h)			
Total AUC	1.87 ± 0.09	2.13 ± 0.09[a]	2.13 ± 0.09[a]
Incremental AUC	-1.11 ± 0.23	-1.36 ± 0.17	-1.36 ± 0.17
3-hydroxybutyrate (µmol.l^{-1}.h)			
Total AUC	170 ± 17	238 ± 17[a,b]	196 ± 17
Incremental AUC	-213 ± 43	-578 ± 247	-255 ± 60
Insulin (µU.l^{-1}.h)			
Total AUC	374.9 ± 42.5	306.0 ± 32.3[a,b]	336.6 ± 38.3[a]
Incremental AUC	294.1 ± 34.9	229.5 ± 27.2[a,b]	261.0 ± 30.6[a]
Glucose (mmol.l^{-1}.h)			
Total AUC	47.8 ± 13.3	49.0 ± 1.0	49.0 ± 1.4
Incremental AUC	2.9 ± 1.1	4.8 ± 0.9[a]	5.1 ± 1.0[a]

N =13, values are mean ± SEM. [a]different from control ($p < 0.05$), [b]different from energy-replacement ($p < 0.05$). AUC: area under the 8.5 h concentration *vs.* time curve, IAUC: incremental AUC, NEFA: non-esterified fatty acids.

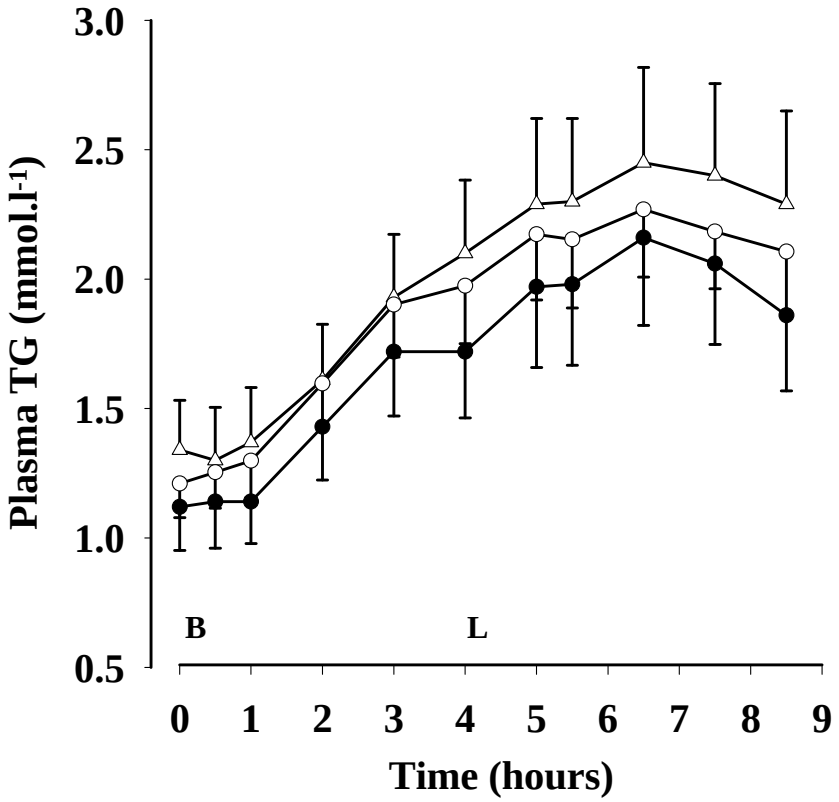


Figure 3.2 Postprandial TG responses for control (Δ), energy-deficit (●) and energy-replacement (o). B and L indicate the times at which the test breakfast (B) and test lunch (L) were provided.

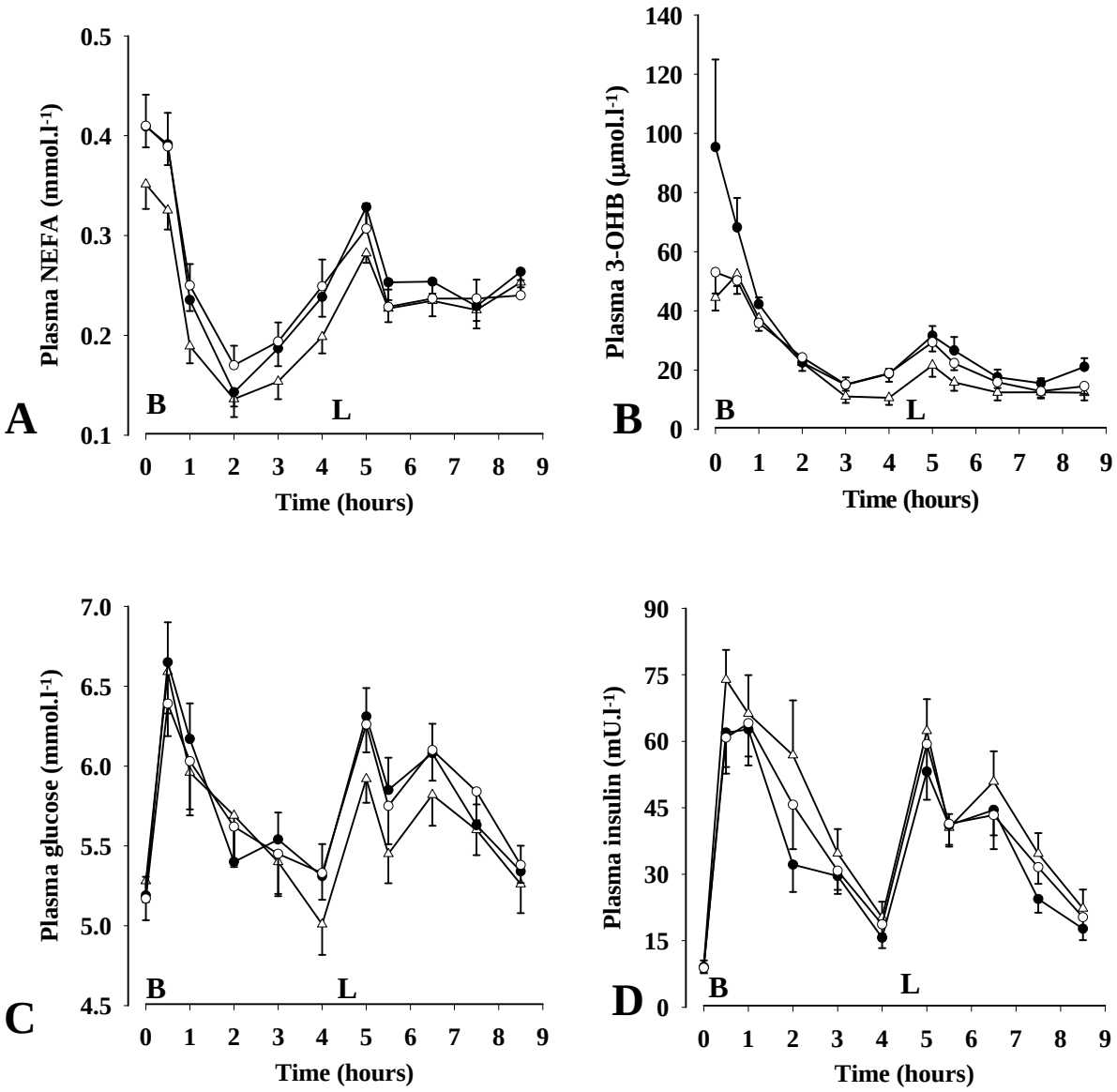


Figure 3.3 Postprandial non-esterified acid (NEFA, A), 3-hydroxybutyrate (3-OHB, B), glucose (g) and insulin (D) responses for control (Δ), energy-deficit (●) and energy-replacement (o). B and L indicate the times at which the test breakfast (B) and test lunch (L) were provided.

The incremental AUC did not differ significantly between trials for the postprandial TG, NEFA and 3-OHB responses. Compared with control, the postprandial glucose incremental AUC was 68% higher and 76% higher for energy-deficit ($p < 0.05$) and energy-replacement ($p < 0.05$), respectively. However, these relatively large percentage differences between trials in the glucose incremental AUC reflected relatively small differences between trials in absolute terms (1.9-2.2 $mmol.l^{-1}.h$ difference in the incremental AUC between the control and energy-deficit and energy-replacement trials, or an average difference over the postprandial observation period of postprandial changes in glucose concentrations of ~0.22-0.26 $mmol.l^{-1}$). The postprandial insulin incremental AUC was 22% lower for energy-deficit ($p < 0.001$) and 11% lower for energy-replacement ($p < 0.01$) compared with control. The insulin incremental AUC was 14% lower in energy-deficit than energy-replacement ($p < 0.01$).

There were no differences in energy expenditure over the 8.5-hour observation period across the three trials. Compared with control, fat oxidation over the postprandial observation period was 30% higher in energy-deficit ($p < 0.001$) and 14% higher in energy-replacement ($p < 0.05$). Postprandial fat oxidation was 12% higher in energy-deficit than energy-replacement ($p < 0.05$). Reciprocally, postprandial carbohydrate oxidation was 18% and 9% lower in energy-deficit ($p < 0.001$) and energy-replacement ($p < 0.05$), respectively, compared with control (**Table 3.3**).

Table 3.3. Postprandial energy expenditure and substrate utilisation (over 8.5 hours)

	Control	**Energy-deficit**	**Energy-replacement**
Respiratory Exchange Ratio	0.87 ± 0.01	0.84 ± 0.01[a,b]	0.85 ± 0.01[a]
Energy Expenditure (kJ)	3114 ± 92	3139 ± 87	3113 ± 79
Fat Oxidation (g)	25.8 ± 1.8	33.5 ± 2.1[a,b]	29.5 ± 1.8[a]
Carbohydrate Oxidation (g)	88.2 ± 4.9	72.4 ± 4.7[a]	79.9 ± 3.9[a]

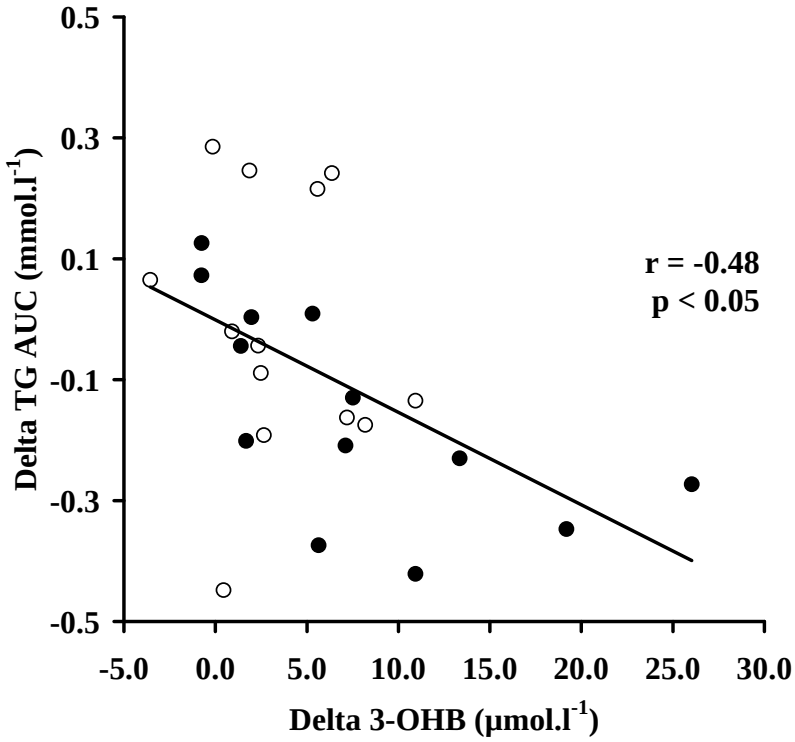
N = 13, values are mean ± SEM and represent total areas under the 8.5 hour variable *vs.* time curve, except for respiratory exchange ratio where the value is reported as the total AUC divided by the duration of observation period (8.5 hours) to give the average postprandial respiratory exchange ratio. [a]different from control ($p < 0.05$), [b]different from energy-replacement ($p < 0.05$)

3.3.3 Relationships between variables within trials

As expected, fasting TG concentrations strongly correlated with the TG total AUC in the control ($r = 0.95$, $p < 0.001$), energy-deficit ($r = 0.98$, $p < 0.001$) and energy-replacement ($r = 0.97$, $p < 0.001$) trials. Fasting TG concentrations significantly correlated with the TG incremental AUC in the energy-deficit ($r = 0.80$, $p < 0.01$) and energy-replacement ($r = 0.76$, $p < 0.01$) trials but not the control trial ($r = 0.42$, $p > 0.05$). There were no significant correlations within trials between any index of TG metabolism (fasting value, total or incremental AUC) and any index of 3-OHB or fat oxidation.

3.3.4 Predictors of the exercise-induced change in TG

A significant correlation was observed between the exercise-induced change in fasting TG concentration and the exercise-induced change in fasting fat oxidation ($r = -0.53$, $p < 0.01$). Significant correlations were also evident between the exercise-induced change in the TG total AUC and the exercise-induced change in the 3-OHB total AUC ($r = -0.48$, $p < 0.05$) and between the exercise-induced change in the TG total AUC and the exercise-induced change in the fat oxidation total AUC ($r = -0.74$, $p < 0.001$) (**Figure 3.4**). Significant correlations were also seen between the exercise-induced change in the 3-OHB total AUC and the change in the fat oxidation total AUC ($r = 0.58$, $p < 0.01$). Multivariate analysis using stepwise regression showed, however, that changes in fat oxidation and 3-OHB total AUC did not predict the change in the postprandial TG total AUC independently of each other, suggesting these relationships were mediated by a common mechanism. No significant correlations were observed between exercise-induced changes in fasting TG and fasting 3-OHB concentrations, or between exercise-induced changes in incremental AUC values for TG, 3-OHB and fat oxidation.



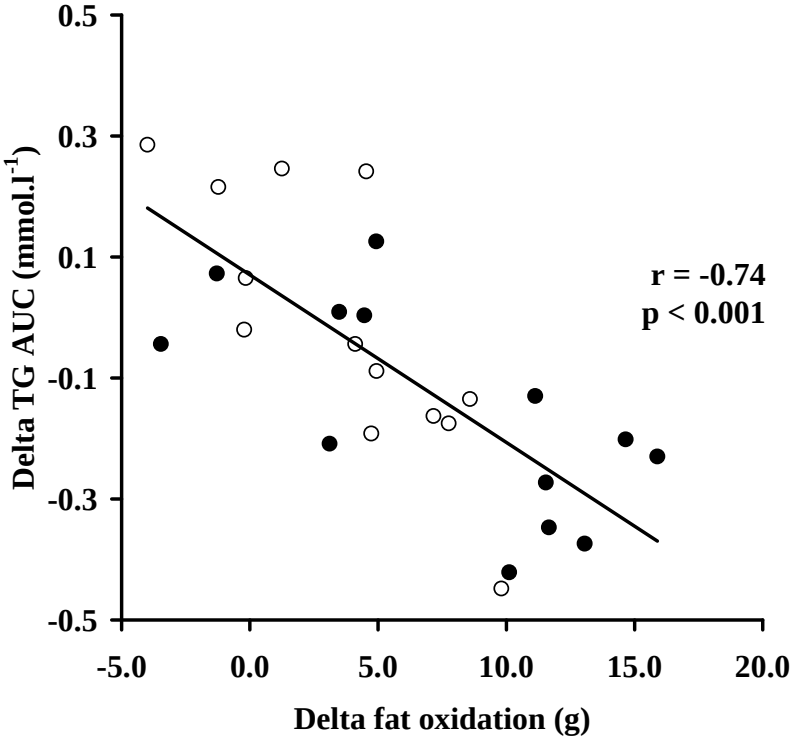


Figure 3.4 The relationship between changes in postprandial triglyceride (TG) and postprandial 3-hydroxybutyrate (3-OHB) responses (top) and between changes in postprandial TG responses and postprandial fat oxidation (bottom) (energy-deficit minus control: ●, energy-replacement minus control: o).

3.4 Discussion

The results of this study demonstrate that a single session of moderate intensity exercise, without an energy deficit, can reduce postprandial insulin concentrations and increase the rate of postprandial fat oxidation. An exercise-induced energy deficit was, however, required to reduce fasting and postprandial TG concentrations and also to induce further lowering of postprandial insulin concentrations and a further increase in postprandial fat oxidation compared to exercise with energy replacement. In addition, these data indicate that changes in postprandial TG concentrations could be explained, in part, by changes in postprandial whole body fat oxidation and postprandial 3-OHB concentrations. Interestingly, while exercise with an energy deficit significantly reduced fasting TG concentrations and postprandial TG total AUC compared with control, it did not significantly reduce the TG incremental AUC. This suggests that the TG-lowering effect elicited by exercise with an energy deficit was predominantly mediated by a decrease in baseline TG concentrations (largely reflecting effects on hepatic VLDL metabolism), with any change in the metabolism of exogenous meal-derived lipids making a much smaller contribution to the TG reduction.

Previous data, reporting that the TG-lowering effect of an exercise-induced energy deficit was greater than that of an equivalent dietary-induced energy deficit, suggested either that the effects of exercise on postprandial TG metabolism were independent of an energy deficit, or that dietary-induced and exercise-induced energy deficits elicited different effects on postprandial metabolism (Gill & Hardman, 2000). Data from the present study, showing that the TG-lowering effect of prior exercise was only evident with an accompanying energy deficit, suggest that the latter interpretation is the correct one. The contrasting effect on TG metabolism of a dietary- or exercise-induced energy deficit may be related to specific body tissues in which the energy deficits occur. During exercise, skeletal muscle glycogen and to a lesser extent, skeletal muscle TG utilisation increases. However, muscle TG utilisation is elevated during the post-exercise recovery period; thus muscle glycogen and triglyceride stores are depleted following exercise (Kiens & Richter, 1998). In contrast, a dietary-induced energy deficit would, at least in the short-term, result in a proportionally greater utilisation of adipose tissue TG. There may also be quantitative differences in hepatic fuel utilisation in response to dietary- and exercise-induced energy deficits. For example, hepatic glycogen content is reduced by about two-thirds following ~80-90 minutes of exercise at 70% $\dot{V}O_2$ max (Casey *et al.*, 2000) which is equivalent to the level of hepatic glycogen depletion following a 24-hour fast (Rothman *et al.*, 1991). Thus, exercise induces quantitatively larger muscle and hepatic substrate deficits than energy

intake restriction. Such substrate deficits in muscle and/or liver may mediate the TG-lowering effects of exercise by stimulating skeletal muscle LPL activity and increasing TG clearance (Gill & Hardman, 2003) and/or by directing the hepatic fatty acid flux towards oxidation and away from re-esterification, thereby reducing VLDL production (Gill *et al.*, 2006b;Gill & Hardman, 2003). Thus greater energy substrate deficits in muscle tissue and liver could mediate the larger effect of exercise-induced, compared to dietary-induced deficits on TG metabolism. This reasoning can also explain why replacement of the exercise-induced energy deficit substantially attenuated the TG-lowering effect. Increased carbohydrate ingestion following exercise markedly increases the rate of both hepatic and muscle glycogen resynthesis (Casey *et al.*, 2000) and thus muscle and hepatic substrate deficits would have been substantially smaller following energy replacement than exercise with energy deficit. In addition, increased carbohydrate ingestion *per se* has been shown to increase fasting and postprandial TG concentrations, probably by increasing hepatic VLDL production (Koutsari *et al.*, 2001), so the increased carbohydrate intake in the exercise with energy replacement trial would have acted to oppose an exercise-induced lowering in TG.

It has previously been reported that exercise-induced reductions in postprandial TG were correlated with exercise-induced increases in the 3-OHB response (Gill *et al.*, 2006a) and hypothesised that this was due to exercise shifting the partitioning of the hepatic fatty acid flux towards β-oxidation and ketone body production and away from re-esterification and VLDL production (Gill *et al.*, 2006b). In the present study a significant correlation between changes in plasma 3-OHB and plasma TG responses was also observed, supporting the earlier hypothesis. Additionally, there were no significant differences in 3-OHB concentrations between the control and exercise with energy replacement trial, suggesting that replacing the energy expended during exercise attenuated the post-exercise up regulation of hepatic fatty acid oxidation. This fits with studies reporting that an increase in post-exercise carbohydrate intake attenuates post-exercise ketogenesis (Koeslag *et al.*, 1982).

In agreement with previous studies (Hansen *et al.*, 2005;Votruba *et al.*, 2002), data from the present study showed an increase, relative to control, in postprandial whole body fat oxidation on the day following exercise with an energy deficit. In addition, exercise with energy replacement also increased postprandial fat oxidation compared to control, albeit to a lesser degree than exercise with an energy deficit. A number of studies have shown that a high respiratory exchange ratio (indicating high carbohydrate and low fat oxidation) measured fasting (Marra *et al.*, 1998;Seidell *et al.*, 1992) or over 24 hours (Zurlo *et al.*,

1990) is a significant predictor of long-term weight gain, independent of metabolic rate (Marra *et al.*, 1998;Seidell *et al.*, 1992;Zurlo *et al.*, 1990). Thus, the present findings raise the possibility that even if energy intake is increased following exercise to replace the exercise-induced energy expenditure, exercise may facilitate long-term weight maintenance through shifts in substrate oxidation and balances. This exciting possibility warrants further investigation and may explain, in part, why regular exercisers gain weight at slower rates than their sedentary peers (Wareham *et al.*, 2005).

In contrast to the effects on postprandial TG concentrations, exercise with energy replacement significantly reduced postprandial insulin concentrations, though to a smaller extent than exercise with an energy deficit. This dissociation between exercise-induced changes in postprandial TG and insulin concentrations has been reported previously (Gill *et al.*, 2002b;Gill *et al.*, 2006a) and suggests that, in the short-term, exercise influences postprandial insulin and TG metabolism by separate mechanisms, although links between insulin sensitivity and TG metabolism are evident over the longer term (Kekalainen *et al.*, 2000;Sinaiko *et al.*, 2006). The smaller effect of exercise on postprandial insulin concentrations when energy was replaced, compared with exercise with an energy deficit, is likely due to increased post-exercise repletion of muscle glycogen stores following the additional food that was provided in this trial, as restricting carbohydrate intake following exercise prolongs the duration of enhanced insulin sensitivity (Cartee *et al.*, 1989). These findings are consistent with long-term exercise training studies which indicate that exercise training without weight or fat loss (suggesting the absence of an exercise-induced energy deficit) improves insulin sensitivity, but that exercise with weight loss induces larger changes (Dengel *et al.*, 1998). Thus, it seems clear that the insulin sensitising effects of exercise are maximised when the energy expended is not replaced by increasing energy intake, however, the extent to which a residual benefit occurs when the energy expended is replaced is unclear.

In summary, the results reported in this study indicate that in overweight or obese but otherwise healthy middle-aged men, exercise with energy replacement lowers postprandial insulin concentrations and increases postprandial fat oxidation. However, the presence of an energy deficit augmented exercise-induced changes in both these factors and an energy deficit was necessary to reduce postprandial TG concentrations. Further study is now needed to establish whether this effect extends beyond the present subject population (e.g. to women, dyslipidemic individuals and those with type 2 diabetes). The experimental model used in the current study, involving two sequential meals with an interval of 4.5

hours and with macronutrient compositions reflecting usual diets consumed in Westernised countries, make the present findings directly applicable to the typical postprandial challenges encountered in every day life. Thus, the findings of the present study indicate that, in overweight and obese men, an exercise session, with the energy expended replaced, can induce some metabolic and cardiovascular benefits, but to maximise the beneficial effects of exercise, an energy deficit is required.

CHAPTER 4

THE EFFECTS OF EXERCISE, WITH AND WITHOUT ENERGY DEFICIT, ON PULSE WAVE VELOCITY IN HEALTHY OBESE MIDDLE-AGED MEN.

4.1 Introduction

Pulse wave velocity (PWV) provides a non-invasive index of endothelial function and arterial stiffness (Asmar *et al.*, 1997;Boutouyrie *et al.*, 2002); it is relatively simple to measure with good repeatability and validity of results (Asmar *et al.*, 1995). The stiffness and distensibility of a blood vessel combine to determine the speed at which the pulse will travel, thus greater stiffness with lower distensibility present faster PWV speeds within that vessel (Asmar *et al.*, 1997). Depending on the measurement site, PWV can be used as a marker of central or peripheral endothelial function and arterial stiffness.

Both endothelial dysfunction and arterial stiffness are considered important risk factors for the development of future cardiovascular events and mortality (Blacher *et al.*, 1999;Boutouyrie *et al.*, 2002). Interventions, such as moderate intensity exercise, which might enhance endothelial function and lower arterial stiffness, may therefore attenuate the risk of cardiovascular disease. It has been reported that endurance trained athletes have lower PWV compared with their sedentary peers, independent of any differences in blood pressure (Otsuki *et al.*, 2007). In addition, prolonged training interventions of 8 weeks and 16 weeks in relatively active (Kakiyama *et al.*, 2005) and sedentary men (Hayashi *et al.*, 2005), respectively, have been associated with a decrease in central PWV, although no effect on peripheral PWV was observed (Hayashi *et al.*, 2005). In the absence of regular physical activity during a two-week detraining period however, central PWV returned to pre-exercise levels (Kakiyama *et al.*, 2005). One limitation with these training studies is, however, that the exercise intervention induced significant reductions in the subjects' body mass. It is therefore unclear as to whether it was the effect of exercise *per se* or the associated weight loss that lowered PWV. It is also not possible to determine whether lower PWV was a true training effect or a response to the last exercise session. To overcome the problem posed by a loss of body mass and or fat mass, and to investigate the acute effect of exercise on PWV, experimental models implementing a single session of exercise can be used to investigate the subsequent effects on PWV, although relatively few studies have used such a model to investigate this acute effect. A maximal treadmill test reduced upper and lower limb PWV by approximately 10% compared with pre-exercise PWV (Naka *et al.*, 2003). Similarly, central and peripheral PWV were lower when

recorded after 30 minutes of moderate cycling exercise in active and sedentary men (Heffernan *et al.*, 2007a;Kingwell *et al.*, 1997b).

Acute exercise may lower PWV by increasing the production and availability of nitric oxide (NO) (Maeda *et al.*, 2004;Roberts *et al.*, 2002), a molecule which induces vasodilatation within a blood vessel (Boger *et al.*, 2003). However, it is also possible that an exercise-induced lowering of PWV may be associated with and determined by subsequent changes in plasma TG concentrations. Plasma TG are correlated with measures of PWV (Legedz *et al.*, 2006;Moritani *et al.*, 1987) and in the postprandial state, changes in central PWV are associated with changes in plasma TG, at least in patients with elevated lipaemia (Daskalova *et al.*, 2005). Following acute moderate intensity exercise plasma TG concentrations are lower (Gill *et al.*, 2002b;Gill & Hardman, 2000;Herd *et al.*, 2001), thus it is plausible to suggest that an exercise-induced lowering in TG could mediate the exercise-induced lowering in PWV. Interestingly, it has been reported that exercise *per se*, may lower TG concentrations via mechanisms which are different to those by which energy deficit attenuates plasma TG (Gill & Hardman, 2000). Therefore it is possible that exercise *per se* may also attenuate PWV values. At this present time however, an association between the exercise-induced change in PWV and TG has not been investigated and the potential role of TG in mediating lower PWV following exercise, remains unclear.

Over recent years, asymmetrical dimethylarginine (ADMA), which is associated with an increase in arterial stiffness (Kielstein *et al.*, 2006), has emerged as a new and strong risk factor for cardiovascular disease (Siroen *et al.*, 2006). ADMA competitively inhibits NO synthase activity (Boger *et al.*, 2003) and reduces NO production (Vallance *et al.*, 1992) and the subsequent vasoconstriction within a vessel and increased arterial stiffness might be reflected in elevated PWV. Evidence supporting a role for exercise in attenuating ADMA concentrations and the risk it poses for CVD is limited. In patients with type I diabetes, ADMA concentrations were lower following two and four months of cycling training (Mittermayer *et al.*, 2005). In patients at risk of coronary disease, 12 weeks of walking or running also reduced ADMA concentrations (Richter *et al.*, 2005) whereas eight weeks of aerobic training in patients with chronic heart failure failed to have any effect (Niebauer *et al.*, 2005). Despite the evidence from these three studies, to the author's knowledge, there has been no attempt to investigate the effects of either prolonged exercise training or a single exercise session on ADMA concentrations in a non-diseased and healthy population. As PWV is attenuated following a single session of moderate

intensity exercise, it is plausible to suggest that a reduction in ADMA might contribute to this effect. This hypothesis does however require testing.

Therefore, there were two main aims of the present study. Firstly, to determine the effects of exercise, with and without energy replacement, on fasting and postprandial PWV and to investigate the association between exercise-induced changes in plasma TG and PWV. Secondly, to determine the effects of acute moderate intensity exercise on plasma ADMA concentrations and to investigate whether changes in PWV may be mediated by changes in ADMA.

4.2 Methods

Thirteen overweight but otherwise healthy men were recruited into this study. Their physical characteristics have previously been described and the reader is referred to Chapter 3.2.

4.2.1 Preliminary sessions

All subjects attended the metabolic suite having fasted for at least 12 hours overnight. After twenty minutes of supine rest, a 25-minute expired air sample was collected to calculate RMR using indirect calorimetry (Chapter 2.5). Daily energy requirements were calculated using a PAL of 1.55 (Chapter 2.4.1.1). Anthropometric measurements were made for each subject as described in Chapter 2.3.

Subjects completed a four-stage sub-maximal fitness test to estimate $\dot{V}O_2$ max and calculate the gradient required to elicit 50% $\dot{V}O_2$ max, the intensity used for the exercise intervention (Chapter 2.7.1). After the fitness test, on a separate occasion, subjects completed a 30 minute treadmill walk which was used to confirm the correct exercise intensity and to estimate the exercise duration required to elicit the target net energy expenditure of 27 $kJ.kg^{-1}$ body mass (Chapter 2.7.2).

4.2.2 Experimental design

Each subject completed three separate two-day trials, performed in a randomised design at least one week apart. Day-one involved one of three interventions: control, exercise with energy deficit (energy-deficit) or exercise with energy replacement (energy-replacement). Day-two was an 8.5 hour metabolic assessment. An overview of the two-day study protocol is given in **Figure 4.1**.

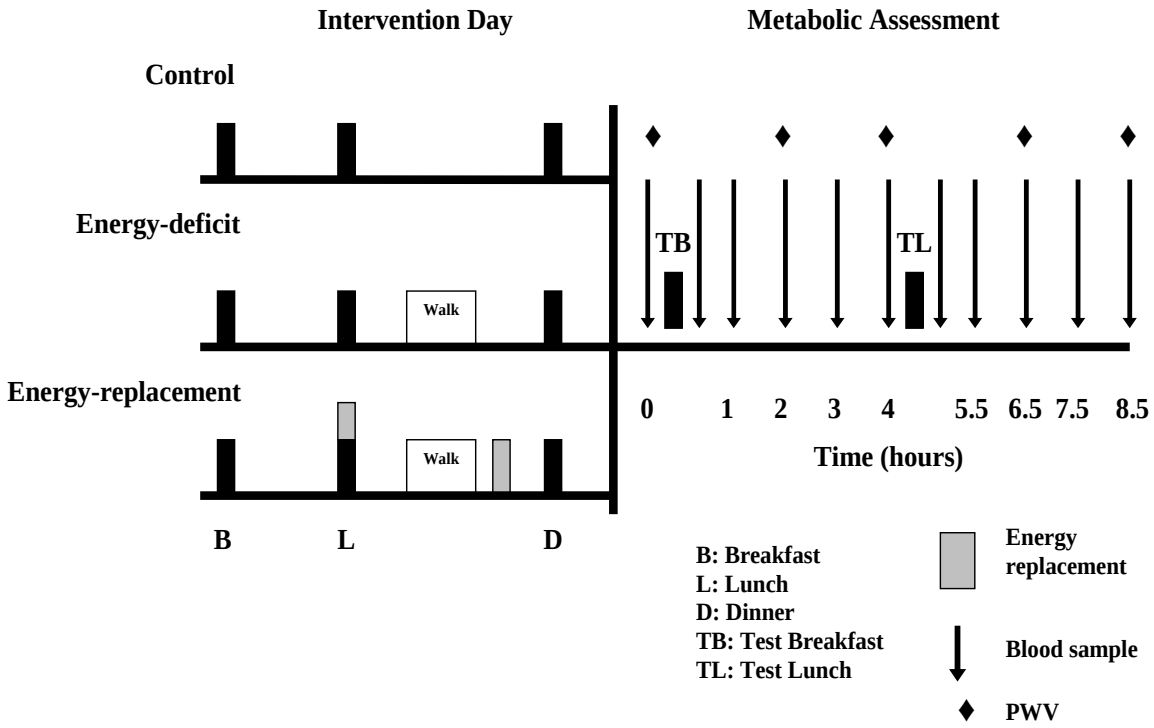


Figure 4.1 An overview of the two-day study design.

Day-one of the control, exercise with energy deficit and exercise with energy replacement trials was completed as described in Chapter 3.2.5.

On Day-two of each trial subjects returned to the metabolic suite after an overnight fast of at least 12 hours, for an 8.5 hour metabolic assessment. Fasting and postprandial blood samples were collected as described in Chapter 2.8.2.1 at 0, 0.5, 1, 2, 3, 4, 5, 5.5, 6.5, 7.5 and 8.5 hours after the test meal provided at breakfast. Pulse wave velocity was measured using the Complior as described in section 2.8.2.2 at 0, 2, 4, 6.5 and 8.5 hours. The test meal (Chapter 2.8.1) was provided immediately after fasting measurements were completed and exactly the same meal was repeated 4.5 hours later.

For the three days prior to Day-two in each experimental trial, all subjects adhered to the pre-trial controls described in Chapter 2.9.

4.2.3 Blood analysis

In each trial, plasma ADMA was determined in the fasting state and at the 8.5 hour time point only. Plasma triglyceride was determined in the fasting state and at regular intervals throughout the postprandial period. A detailed description of these analysis is given in Chapter 2.10.

4.2.4 Data analysis

The total AUC, calculated using the trapezium rule, divided by the duration of the observation period (8.5 hours) i.e. the time-averaged AUC, was used as a summary measure of the PWV and TG responses.

Data were analysed using Statistica (version 6.0, StatSoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennsylvania). Prior to analysis, all data were tested for normality using the Anderson-Darling normality test and, if necessary, logarithmically transformed. Only TG concentrations were transformed prior to statistical analysis. Differences in fasting and summary postprandial responses for TG and PWV were analysed using repeated measures one-way ANOVA. Differences over time for the three trials were calculated using two-way ANOVA with repeated measures for trial and time. Post hoc Fisher least significant difference tests were used to identify where differences lay when main trial and or interaction effects were observed. Between trial differences in fasting and 8.5 hour ADMA concentrations were analysed using repeated measures ANOVA. Within trial differences between fasting and 8.5 hour ADMA measurements were analysed using paired t-tests. Relationships between variables were assessed using Pearson product-moment correlations. Statistical significance was accepted at the $p < 0.05$ level and data are presented as mean ± SEM, unless otherwise stated.

4.3 Results

4.3.1 Responses during the treadmill walk

In both the energy-deficit and energy-replacement treadmill walks, subjects walked for 90.8 ± 3.6 minutes at a speed of 5.5 ± 0.1 $km.h^{-1}$ up a gradient of 3.4 ± 0.5%. Mean $\dot{V}O_2$ was 19.1 ± 0.6 $ml.kg^{-1}.min^{-1}$ and 19.3 ± 0.6 $ml.kg^{-1}.min^{-1}$ for energy-deficit and energy-replacement, respectively. Mean heart rate was 122 ± 3 $beat.min^{-1}$ and 123 ± 3 $beat.min^{-1}$ for energy-deficit and energy-replacement, respectively. Both treadmill walks were tolerated well with subjects rating the walk as " fairly light" on the Borg scale of 6-20 (Borg, 1973). Net energy expenditure was 2.80 ± 0.12 MJ for energy-deficit and 2.80 ± 0.12 MJ for energy-replacement. There were no significant differences between the two trials in any of the above variables.

4.3.2 Metabolic assessment: Responses in the fasted state

A summary of all fasting responses is given in **Table 4.1.** Compared with control, fasting PWV was 9% and 7% lower for energy-deficit ($p < 0.05$) and energy-replacement ($p < 0.05$), respectively. Fasting PWV was not significantly different between the two exercise

trials ($p > 0.05$). Fasting TG concentrations were 16% lower in energy deficit compared with control but no significant difference was observed in fasting TG concentrations between energy-replacement and control ($p > 0.05$) or energy deficit and energy-replacement ($p > 0.05$). Neither fasting heart rate nor fasting ADMA concentrations were significantly different between trials ($p > 0.05$ for both).

Table 4.1 Fasting plasma and metabolic values

	Control	**Energy-deficit**	**Energy-replacement**
PWV* ($m.s^{-1}$)	10.15 ± 0.36	9.28 ± 0.33[a]	9.40 ± 0.31[a]
Triglyceride ($mmol.l^{-1}$)	1.34 ± 0.19	1.12 ± 0.17[a]	1.21 ± 0.13
Heart rate (bpm)	59 ± 3	58 ± 4	56 ± 3
ADMA ($\mu mol.l^{-1}$)	0.63 ± 0.04	0.66 ± 0.04	0.64 ± 0.04

N = 13. Values are mean ± SEM. [a]different to control ($p < 0.05$). PWV: pulse wave velocity, ADMA: asymmetrical dimethylarginine. * PWV was measured between the carotid and radial sites.

4.3.3 Metabolic assessment: Responses in the postprandial state

A summary of the postprandial PWV, TG and heart rate responses is given in **Table 4.2.** **Figure 4.2** shows the postprandial PWV response over the 8.5 hour metabolic assessment day. **Figure 4.3** shows the mean time-averaged PWV speed for each of the three trials. The time-averaged PWV was 0.55 $m.s^{-1}$ (5%) and 0.65 $m.s^{-1}$ (6%) slower in energy-deficit and energy-replacement, respectively, compared with control ($p < 0.05$ for both). There was no significant difference in PWV between the two exercise trials ($p > 0.05$). Postprandial PWV was significantly faster at 8.5 hours compared to fasting in energy-deficit ($p < 0.01$) and energy-replacement ($p < 0.01$) but not control ($p > 0.05$). The time-averaged postprandial TG response was 14% lower in energy-deficit compared with control ($p < 0.05$) and 10% lower in energy-deficit compared with energy-replacement ($p < 0.05$). The postprandial TG response was 5% lower in energy-replacement compared with control but this did not reach significance ($p > 0.05$). Time-averaged postprandial heart rate did not differ between trials ($p > 0.05$). The incremental AUC did not differ significantly between trials for the postprandial PWV, TG or heart rate responses. **Figure 4.4** shows the mean ADMA concentration for each trial measured at 8.5 hours. No significant differences in the postprandial plasma ADMA concentrations were observed between the three trials (control: 0.66 ± 0.04 $\mu mol.l^{-1}$, energy-deficit: 0.74 ± 0.15 $\mu mol.l^{-1}$, energy-replacement: 0.67 ± 0.04 $\mu mol.l^{-1}$, $p > 0.05$ for all).

Table 4.2 Postprandial plasma and metabolic responses

	Control	Energy-deficit	Energy-replacement
PWV* ($m.s^{-1}$)			
Time-averaged AUC	10.28 ± 0.37	9.73 ± 0.29[a]	9.63 ± 0.28[a]
Incremental AUC	0.13 ± 0.08	0.45 ± 0.21	0.23 ± 0.13
Triglyceride ($mmol.l^{-1}$)			
Time-averaged AUC	2.01 ± 0.28	1.73 ± 0.26[a,b]	1.90 ± 0.21
Incremental AUC	0.67 ± 0.11	0.60 ± 0.10	0.69 ± 0.09
Heart rate (bpm)			
Time-averaged AUC	60.0 ± 3.1	60.0 ± 3.4	59.0 ± 3.4
Incremental AUC	0.7 ± 0.9	2.0 ± 1.0	2.8 ± 1.0

N = 13. Values are mean ± SEM. [a]different to control ($p < 0.05$), [b]different to energy-replacement ($p < 0.05$). AUC: area under the 8.5 hour concentrations *vs.* time curve divided by the duration of the assessment period (8.5 hours), PWV: pulse wave velocity. * PWV was measured between the carotid and radial sites.

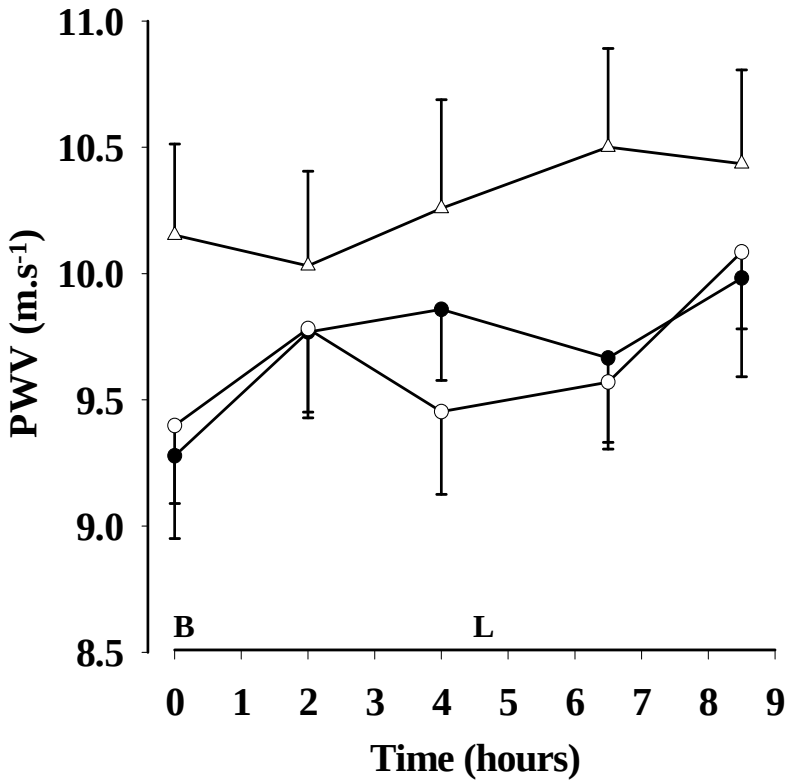


Figure 4.2 Postprandial PWV response for control (Δ), energy-deficit (●) and energy-replacement (o). B and L indicate the times at which the test breakfast (B) and test lunch (L) were provided.

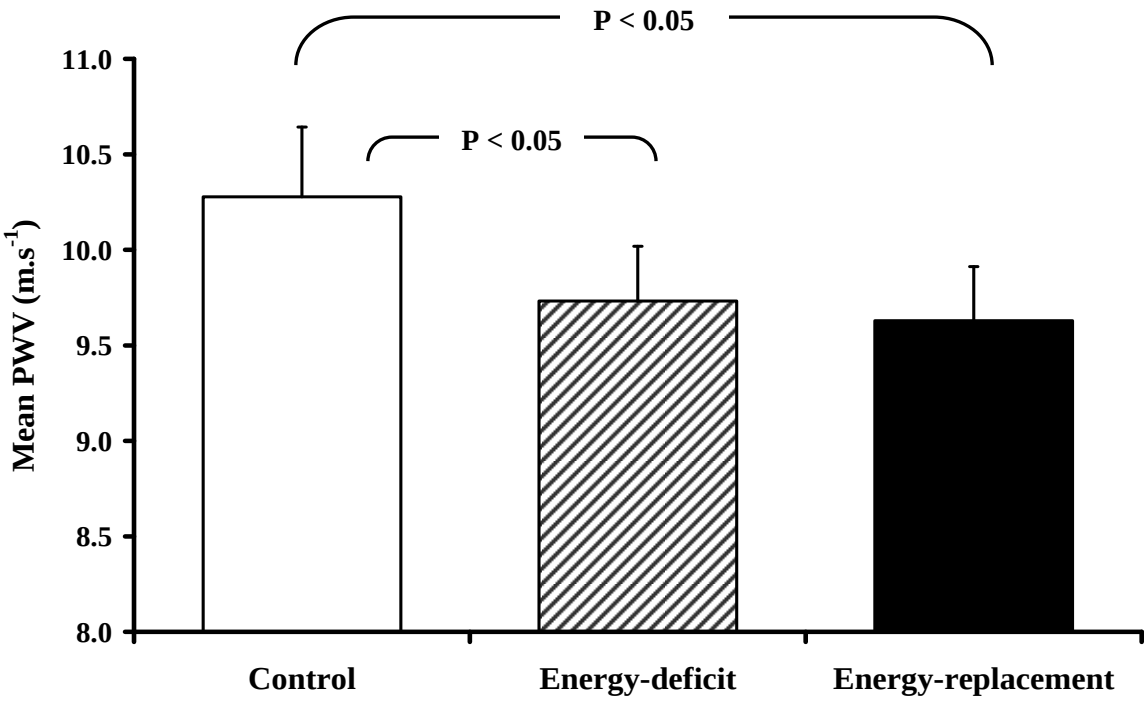


Figure 4.3 Time-averaged postprandial PWV for control, energy-deficit and energy-replacement.

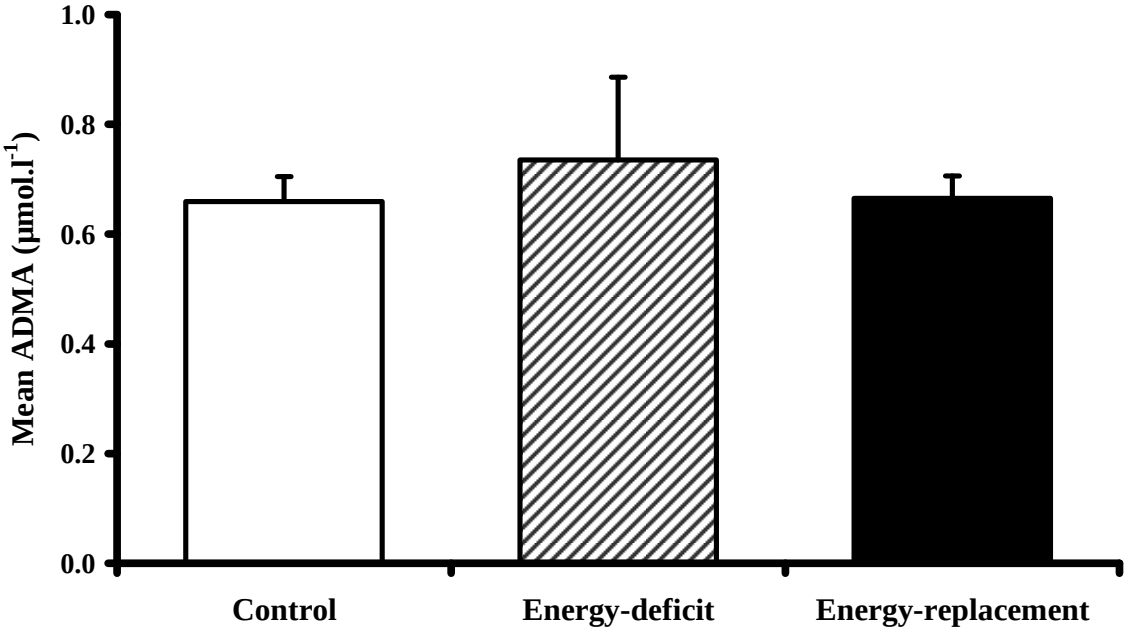


Figure 4.4 Postprandial ADMA concentrations measured at 8.5 hour for control, energy-deficit and energy-replacement.

4.3.4 Relationships between variables

No correlation was observed between the fasting or postprandial PWV responses and plasma ADMA concentrations, systolic BP, diastolic BP, BMI or waist circumference. Significant correlations were observed between fasting PWV and fasting TG concentrations ($r = 0.57$, $p < 0.001$), as shown in **Figure 4.5,** with individual correlations of 0.51, 0.63 and 0.54 for control, energy-deficit and energy-replacement, respectively. Significant correlations were also observed between postprandial PWV and postprandial TG concentrations ($r = 0.46$, $p < 0.01$), as shown in **Figure 4.6,** with individual correlations of 0.44, 0.50 and 0.47 for control, energy-deficit and energy-replacement, respectively. Exercise-induced changes in fasting PWV were correlated with the exercise-induced changes in postprandial PWV ($r = 0.87$, $p < 0.001$). Exercise-induced changes in fasting PWV were also correlated with exercise-induced changes in fasting TG concentrations ($r = 0.44$, $p < 0.05$) as shown in **Figure 4.7.** No correlation was observed between the exercise-induced changes in postprandial PWV responses and exercise-induced changes in postprandial TG concentrations ($r = 0.26$, $p > 0.05$).

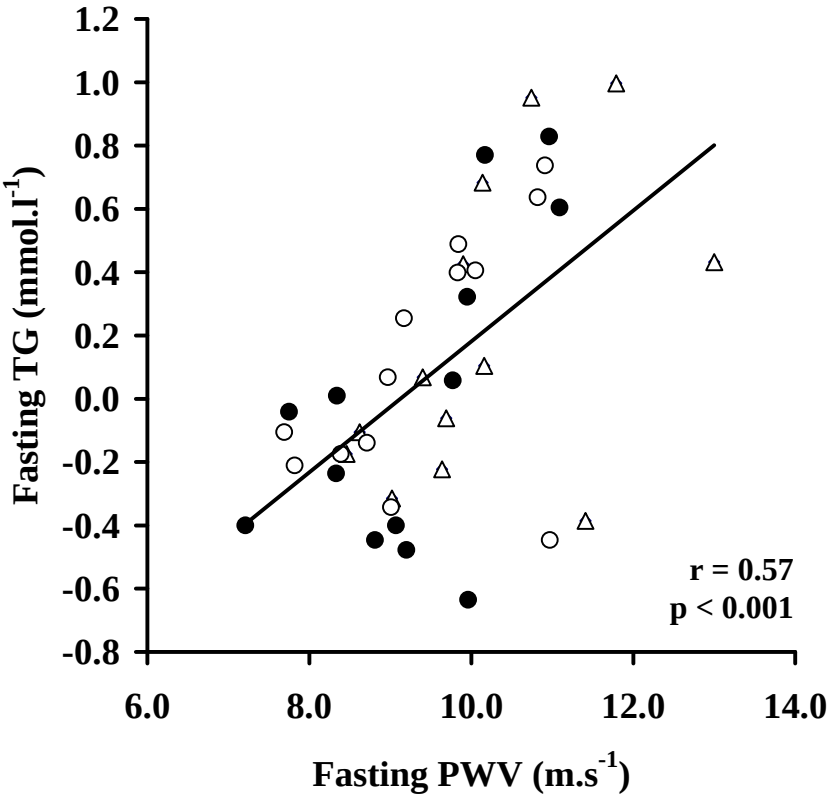


Figure 4.5 The relationship between fasting PWV and fasting TG concentrations in control (Δ), energy-deficit (●) and energy-replacement (O).

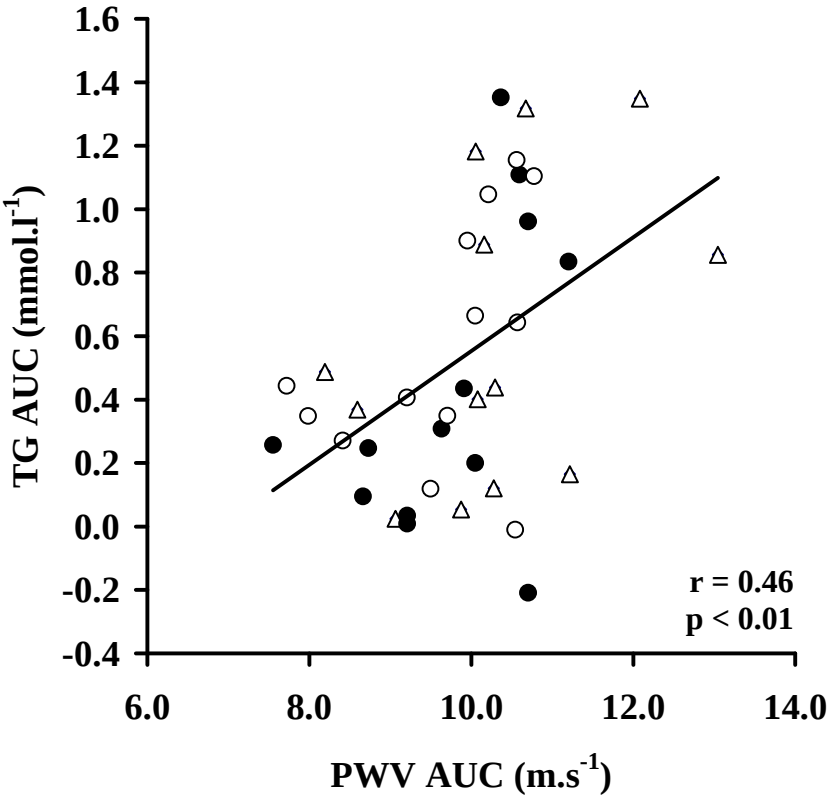


Figure 4.6 The relationship between postprandial PWV and postprandial TG responses in control (Δ), energy-deficit (●) and energy-replacement (O).

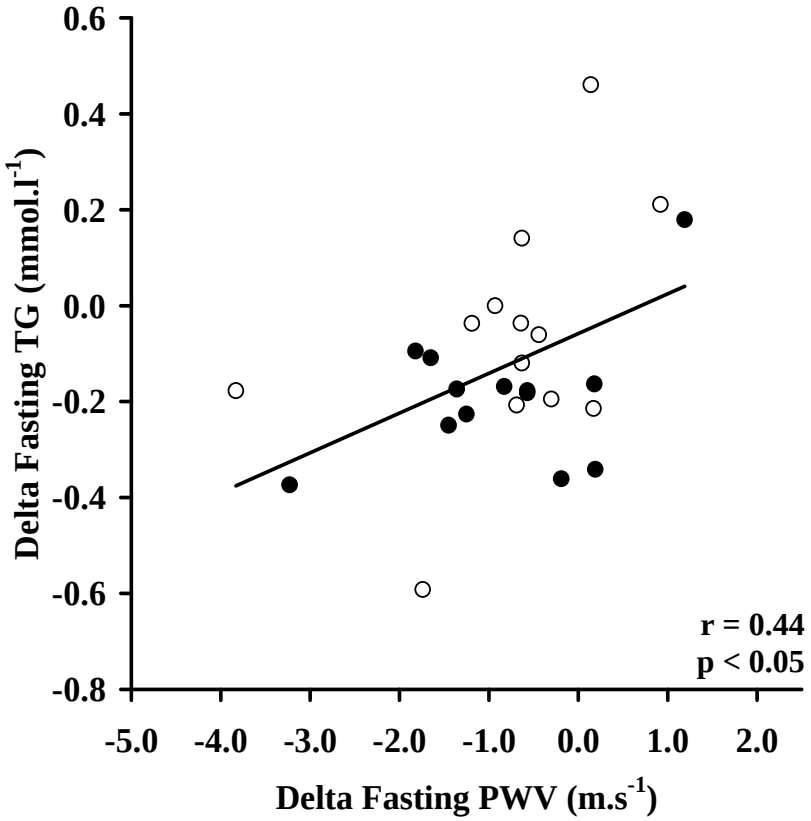


Figure 4.7 The relationship between the exercise-induced changes in fasting PWV and fasting TG responses (energy-deficit minus control: ●, energy-replacement minus control: o).

4.4 Discussion

The results of this study demonstrate that a single session of moderate intensity exercise lowers peripheral PWV independently of the associated exercise-induced energy deficit. The same moderate intensity exercise, however, had no effect on either fasting or postprandial ADMA concentrations, suggesting that the exercise induced lowering of PWV, and therefore improved endothelial function and lower arterial stiffness, was independent of any change in plasma ADMA.

Peripheral PWV was measured using the Complior, a non-invasive and reliable method for assessing endothelial function and arterial stiffness of blood vessels (Asmar *et al.*, 1995). Thirty minutes of moderate intensity cycling has previously been shown to lower both central and peripheral PWV measured 20-30 minutes post exercise in both active (Heffernan *et al.*, 2007a) and sedentary men (Kingwell *et al.*, 1997b). High intensity treadmill exercise lowers peripheral PWV, measured in both the arms and legs, after 60

minutes of recovery in sedentary men and women (Naka *et al.*, 2003). Data from the present study adds to these findings by showing that moderate intensity walking lowers peripheral PWV in obese middle-aged men and that these effects are evident on the day following exercise. Furthermore, exercise significantly lowered PWV independently of the exercise-induced energy expenditure and subsequent energy deficit. Interestingly, whilst both energy-deficit and energy-replacement significantly lowered the fasting and postprandial PWV responses, there was no effect of either exercise trial on the incremental AUC. This suggests that the postprandial lowering of PWV following exercise is most likely mediated by the changes in fasting PWV *per se* rather than changes in the postprandial response.

A novel finding of the present study showed PWV to be lower the day after 90 minutes of treadmill walking. The author is unaware of any other studies that have reported such a prolonged effect of acute exercise on PWV, with previous literature measuring PWV only immediately after or during a relatively short post exercise recovery period of up to four hours (Clegg *et al.*, 2007;Heffernan *et al.*, 2007a;Kingwell *et al.*, 1997b;Naka *et al.*, 2003). The duration of the exercise effect on PWV in these previous studies has been varied. Thirty minutes of cycling significantly lowered central and peripheral PWV compared to pre-exercise values at 30 minutes post exercise but not after 60 minutes of recovery, suggestive of a very short term effect of exercise (Kingwell *et al.*, 1997b). Naka et al. (Naka *et al.*, 2003) reported that at 60 minutes post exercise, peripheral PWV remained 10% lower than pre-exercise values. In their study however, PWV was not measured beyond 60 minutes so the total duration of the exercise effect on PWV is not known. It is possible that if they had continued to measure PWV, Naka et al. would have observed a more prolonged attenuation of PWV, and indeed Clegg *et al*. did report lower peripheral PWV four hours post-exercise. In contrast, the present study reported peripheral PWV to be 9% and 7% lower when measured approximately 16 hours after the energy-deficit and energy-replacement exercise interventions, respectively. Although these findings are somewhat conflicting with those of Kingwell *et al*. (Kingwell *et al.*, 1997b), they are consistent with the literature that has used other methods to measure endothelial function and reported improved endothelial function the day following moderate intensity exercise (Gill *et al.*, 2004). A number of reasons might explain the different results from this study and those of Kingwell's group. The exercise intervention implemented by both groups was quite different with the present study using a 90 minute moderate intensity treadmill walk and Kingwell *et al*. using a 30 minute moderate intensity cycling session (Kingwell *et al.*, 1997b). Although the present study has shown exercise to lower PWV independently of

the associated energy deficit, it is possible that the larger energy-expenditure associated with 90 as opposed to 30 minutes of exercise contributed to the greater reduction in PWV. The duration *per se* of the two exercise protocols might also explain the differences. It has been suggested that the role of NO in mediating vasoactivity within a vessel increases with the duration of exercise (Maiorana *et al.*, 2003). Considering that subjects in the present study exercised for 60 minutes longer than those in the study of Kingwell et al. this might explain the prolonged effect on PWV that was observed some 16 hours post exercise. It might also explain why Naka *et al*. (Naka *et al.*, 2003) reported PWV to be lower at 60 minutes of recovery after an hour of exercise whereas Kingwell et al. (Kingwell *et al.*, 1997b) reported no such effect. The subject populations used in these two studies should also be considered. For the current study, middle-aged obese men were recruited whereas Kingwell *et al*. studied young and lean men. It is possible that exercise induces a different and perhaps greater effect on PWV in obese subjects, a population already at higher risk of impaired endothelial function (Caballero, 2003), compared to normal weight subjects. The absolute reduction in plasma TG following a session of moderate intensity exercise is greater in obese subjects compared to lean controls (Gill *et al.*, 2004). Considering the evidence for a role of TG in mediating PWV, this might explain the different effects of exercise on PWV in this study compared to Kingwell *et al.* (Kingwell *et al.*, 1997b). Further research to determine whether the type and duration of the exercise intervention and also the subject population can influence the effect of exercise on PWV is warranted.

The mechanism for the lowering of PWV cannot be elucidated from the measurements made in the present study. Changes in the structure and functional properties of blood vessel walls have been observed with prolonged exercise training (Kingwell *et al.*, 1997a), however such a mechanism is unlikely to explain the acute changes observed after a single session of exercise. A more likely explanation for lower PWV speeds is related to the effects of exercise on NO, a molecule stimulating vasodilation in a blood vessel (Boger *et al.*, 2003) which is known to increase in concentration following exercise (Jungersten *et al.*, 1997;Roberts *et al.*, 2002;Tozzi-Ciancarelli *et al.*, 2002). Greater blood flow following exercise (Malkova *et al.*, 2000) and the shear stress this induces on the blood vessel may further contribute to the lower PWV observed the day following exercise.

In the present study, ADMA was also investigated as a potential mechanism to explain the exercise-induced lowering of PWV. ADMA is a known inhibitor of NO synthase (Boger *et al.*, 2003) and associated with increased levels of arterial stiffness (Kielstein *et al.*, 2006). A reduction in plasma ADMA following exercise could therefore restrict the

inhibitory effect of ADMA on NO synthase thus promoting NO production. Such a mechanism has however received little attention from research groups and very few data regarding the effect of exercise on ADMA are available. Aerobic training lowers ADMA concentrations in patients with type I diabetes (Mittermayer *et al.*, 2005) and patients at risk of coronary heart disease (Richter *et al.*, 2005) but findings are equivocal, with no effect of exercise training on ADMA observed in patients with chronic heart failure (Niebauer *et al.*, 2005). Training programmes comprise regular episodes of acute exercise and it is possible that lower ADMA concentrations observed with training are in fact a consequence of acute exercise *per se*. To the best of the author's knowledge, there have however been no studies investigating the effects of acute exercise on ADMA, therefore it is shown here for the first time that a single session of moderate intensity exercise does not attenuate either fasting or postprandial plasma ADMA concentrations, at least on the day following exercise, suggesting that ADMA is unlikely to contribute to lower PWV following exercise reported here and also by others (Heffernan *et al.*, 2007a;Kingwell *et al.*, 1997b;Naka *et al.*, 2003).

It is not apparent why acute exercise failed to show any effect on plasma ADMA concentrations whereas Mittermayer *et al*. (Mittermayer *et al.*, 2005) and Richter *et al*. (Richter *et al.*, 2005) both reported lower ADMA concentrations after exercise training. It could be that changes in ADMA occur only with regular training and a single session of exercise is insufficient to induce any changes. It is possible that by measuring plasma ADMA only in the fasting state and 8.5 hours postprandially on the day following exercise, any immediate effect of exercise on ADMA concentrations has been overlooked and this should perhaps be investigated in future studies. Differences in subject population may explain our different results; we recruited obese but otherwise healthy men whereas relatively diseased populations have been used in training studies (Mittermayer *et al.*, 2005;Richter *et al.*, 2005). If exercise regulates ADMA differently in healthy and diseased individuals it might explain why the present study failed to find any effect of exercise compared to others. It should be considered however that of the three training studies that have been published, one failed to report any effect of exercise on ADMA, consistent with the findings from the present study. It is both the lack of data and inconsistent findings that makes it somewhat difficult to determine the true effect of acute and prolonged exercise on ADMA. Future research is justified to clarify whether the effects of exercise on endothelial function and arterial stiffness are indeed mediated by ADMA and if they are, what the mechanism is that drives such an effect.

In the present study, a significant positive correlation was observed between PWV and plasma TG concentrations in both the fasted and postprandial state, findings which are consistent with the literature (Legedz *et al.*, 2006;Moritani *et al.*, 1987) and suggest that higher plasma TG concentrations are related to higher and therefore faster PWV speeds. Daskalova *et al*. (Daskalova *et al.*, 2005) also showed postprandial changes in PWV to be correlated with postprandial changes in TG concentrations. In this study, there was no apparent relationship between the exercise-induced changes in postprandial PWV and TG responses, however, the exercise-induced changes in fasting PWV and TG concentrations were correlated. As suggested above, the postprandial changes in PWV were mediated by changes in fasting PWV, shown by the absence of any differences in the PWV incremental AUC between the three trials. Therefore, it is likely that the exercise-induced reduction in fasting TG concentrations mediated the lower fasting and therefore lower postprandial PWV responses observed after exercise. Overall, 19% of the variance in fasting PWV was explained by the changes in plasma TG, which is significant but relatively small, suggesting that other mechanisms are also likely to be important in the regulation of PWV the day after exercise. Increased TG have previously been associated with impaired endothelial function (Lewis *et al.*, 1999;Lundman *et al.*, 1997;Vogel *et al.*, 1997) and a reduction in endothelium-dependant vasoactivity (Lundman *et al.*, 1997;Vogel *et al.*, 1997), thus data from this study supports a role for the exercise-induced lowering of fasting TG concentrations in attenuating PWV, which is indicative of enhanced endothelial function and decreased arterial stiffness. How TG directly impairs endothelial function is not clear although one proposed mechanism is that TG both within and subintimal to the endothelium of a blood vessel in some way restricts the diffusion of NO across the endothelium. Subsequently, NO becomes degraded more readily and before it is able to induce vasodilatation of that blood vessel (Lundman *et al.*, 1997).

One consideration regarding the present study is that the Complior was used to measure PWV which only provides a marker of endothelial function and arterial stiffness not a direct measurement. However, measurement of PWV using the Complior has been shown to be a valid and reliable method for assessing endothelial function (Asmar *et al.*, 1995) and the author is satisfied that this is a viable method to use for determining the effects of exercise, with and without energy replacement, on endothelial function and arterial stiffness. A second consideration is that blood pressure was not measured on the individual trial days. Blood pressure is related to PWV (Asmar *et al.*, 1997) and it could be argued that lower blood pressure following exercise may have contributed to the lower fasting and postprandial PWV observed the day after the energy-deficit and energy-

replacement treadmill walks. Post-exercise hypotension has been reported following acute exercise although the duration of such an effect appears variable. Some report exercise-induced hypotension to be short lived, lasting for just one hour of exercise recovery (MacDonald *et al.*, 2000;Somers *et al.*, 1991) however, others have reported the effect to persist for 12 hours or more (Pescatello *et al.*, 1991;Wallace *et al.*, 1999). It is therefore not possible to eliminate any regulatory effect that changes in blood pressure may have had on the exercise-induced attenuation of peripheral PWV reported in the present study. Measurement of blood pressure in future studies will hopefully elucidate the role that exercise-induced changes in blood pressure may have for mediating the PWV response.

In summary the results of the present study indicate that in obese but otherwise healthy middle-aged men, moderate intensity exercise significantly lowers fasting and postprandial peripheral PWV (a marker of arterial stiffness and endothelial function) independently of the exercise-induced energy deficit, when in a state of energy balance. Therefore, individuals who exercise but fail to lose body weight, possibly due to increasing their food intake (Stubbs *et al.*, 2002b), may still incur significant improvements to endothelial function and lower arterial stiffening. It is possible that exercise-induced changes in PWV are mediated, at least in part, by exercise-induced changes in plasma TG concentrations whilst exercise-induced changes in plasma ADMA do not seem to have an effect on PWV. Additional research is now needed to confirm that these findings can be extended to other subject populations such as women, patients with type 2 diabetes and those with dyslipidemia.

CHAPTER 5
EFFECTS OF EXERCISE, WITH AND WITHOUT AN ENERGY DEFICIT, ON POSTPRANDIAL LEPTIN AND GHRELIN CONCENTRATIONS AND APPETITE RESPONSES.

5.1 Introduction

Tighter coupling between energy expenditure and food intake may explain why regular exercisers are better able to maintain stable body weights and are less susceptible to weight gain (King *et al.*, 1997b;Wareham *et al.*, 2005). Evidence for any change in appetite sensations following exercise does however remain inconclusive (Broom *et al.*, 2007;Hubert *et al.*, 1998;Imbeault *et al.*, 1997;Tsofliou *et al.*, 2003;Westerterp-Plantenga *et al.*, 1997a). A greater understanding of the exercise-induced changes in appetite and leptin and ghrelin, hormones mediating both long and short term regulation of energy balance may offer further insight into how appetite and body weight might be regulated by exercise.

Leptin, a hormone secreted from adipose tissue, controls energy balance and body mass via its regulation of food intake and energy expenditure (Friedman & Halaas, 1998). In rodent models, leptin administration lowers food intake, reduces body weight and increases physical activity. However the effect in humans remains unclear (Jequier, 2002). Leptin is positively associated with various markers of adiposity such as fat mass, BMI, waist circumference and percentage body fat (Abdella *et al.*, 2005;Considine *et al.*, 1996;Hickey *et al.*, 1996b) with obese individuals presenting elevated leptin profiles compared to their normal weight peers (Considine *et al.*, 1996) which indicates some degree of leptin resistance (Dyck, 2005). Weight loss interventions show a decline in circulating leptin (Considine *et al.*, 1996) whilst an increase in leptin is observed after weight gain (Kolaczynski *et al.*, 1996b), findings which suggest a role for leptin in regulating the size of adipose tissue mass (Friedman & Halaas, 1998). However, significant changes in leptin are also observed after relatively short periods of fasting (Kolaczynski *et al.*, 1996a), energy restriction (Mars *et al.*, 2005) or excess overfeeding (Kolaczynski *et al.*, 1996b;Kolaczynski *et al.*, 1996a), during which time minimal weight loss or gain is observed. These data suggest that leptin is regulated by and responds to changes in energy balance status rather than adipose tissue mass *per se*.

Leptin concentrations, both in the fasted (Keller *et al.*, 2005;Tuominen *et al.*, 1997) and postprandial state (Duclos *et al.*, 1999), are attenuated in response to acute exercise, again

suggesting a role for energy balance status in regulating leptin. These findings are though, by no means unequivocal and some studies have failed to find any effect of exercise on leptin (Perusse *et al.*, 1997;Racette *et al.*, 1997;Tsofliou *et al.*, 2003). It is possible that the absence of any change in leptin in these studies was a consequence of their using a single leptin measurement made during the immediate post exercise period, which was likely to have overlooked the delayed effect of exercise on lowering leptin (Essig *et al.*, 2000). The moderate amount of energy expended during exercise interventions could also explain the absence of any change in leptin in previously published reports. It is widely believed that only large exercise-induced energy expenditures and energy deficits are successful in lowering plasma leptin concentrations (Considine, 1997), although some evidence is available to suggest that energy expenditure and energy deficit alone may not mediate leptin responses. Essig *et al*. (Essig *et al.*, 2000) reported fasting leptin to be reduced by 30% following exercise with an associated energy expenditure of either 800 kcals or 1500 kcals, suggesting that a greater energy expenditure did not facilitate any greater reduction in leptin concentrations. Another well designed study by van Aggel-Leijssen *et al*. (Aggel-Leijssen *et al.*, 1999) compared 24-hour leptin profiles following a period of exercise or rest in a state of energy balance or positive energy balance. They reported plasma leptin to be significantly lower following exercise in a state of energy balance compared with rest in energy balance. Findings from these two studies suggest that factors other than energy balance status mediate the leptin response. It should be highlighted, however, that Hilton and Loucks (Hilton & Loucks, 2000) failed to report any change in 24-hour leptin profiles following a period of exercise either in energy balance or energy deficit. At present, therefore, the role of energy balance status in mediating the exercise-induced lowering of plasma leptin concentrations remains unclear. It might be that rather than energy balance *per se*, leptin responds to exercise-induced changes in energy substrate, i.e. fat and carbohydrate, availability (Hilton & Loucks, 2000), however such a hypothesis requires further investigation.

A role for leptin in the short term regulation of energy balance, possibly via the secretion of gastric leptin and its interaction with other hormones (Friedman & Halaas, 1998;Klok *et al.*, 2007), has also been proposed. At rest, however, leptin concentrations do not change in response to food intake (Jequier, 2002) and associations between leptin, satiety and appetite are also unclear (Di, V *et al.*, 2006;Heini *et al.*, 1998;Mars *et al.*, 2006;Tsofliou *et al.*, 2003). It is therefore, perhaps not surprising that any place for leptin in controlling energy balance in the short term has been questioned (Jequier, 2002). Interestingly, one study has shown that although no association between leptin and appetite was observed at

rest and after food, following a single exercise session leptin correlated with hunger, satiety, prospective food consumption (PFC), fullness and desire to eat (Tsofliou *et al.*, 2003), suggesting that exercise might induce a tighter coupling between leptin and appetite measures. Surprisingly however, to the best of the author's knowledge, there is little other research available investigating such a relationship between leptin and appetite post exercise, thus the role for leptin in modulating short term appetite control requires further clarification.

Ghrelin is released from the stomach in response to the current nutrient status (Klok *et al.*, 2007). Unlike leptin, ghrelin is orexigenic, stimulating rather than suppressing appetite (Wren *et al.*, 2001) and ghrelin is also a key player in the short term regulation of energy balance (Klok *et al.*, 2007). Infusion of ghrelin increases ratings of hunger and food intake in both animals (Wren *et al.*, 2000) and humans (Druce *et al.*, 2005;Wren *et al.*, 2001). A number of studies have investigated the effects of acute exercise on plasma ghrelin concentrations, most of which have reported that exercise lasting anywhere from 10 minutes to one hour has no effect on the ghrelin response (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a). One study did however report significantly lower ghrelin concentrations after maximal exercise (Vestergaard *et al.*, 2007), although another study reported significantly higher ghrelin concentrations immediately after maximal rowing (Jurimae *et al.*, 2007b). There does, therefore, remain some uncertainty surrounding the effect of acute exercise on plasma ghrelin. Whether ghrelin is associated with subjective ratings of appetite and satiety post exercise also remains unclear. To the best of the author's knowledge, only one study has investigated the relationship between ghrelin and appetite following exercise (Burns *et al.*, 2007) in which it was reported that exercise-induced changes in hunger were not related to changes in plasma ghrelin concentrations, however, such a relationship must be further investigated.

Despite the majority of the evidence suggesting that exercise has no effect on plasma ghrelin (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a) and that ghrelin does not mediate appetite control (Burns *et al.*, 2007), it is important to consider that these studies have recruited healthy, normal weight subjects. In overweight and obese individuals appetite is likely to be less tightly regulated (Herman & Polivy, 2005), causing them to consume more calories than required and gain excess weight. It is important to determine if exercise has any effect on plasma ghrelin in the obese and also whether exercise might modulate the relationship between plasma

ghrelin concentrations and appetite in this population. Furthermore, in the literature, the effects of acute exercise on plasma ghrelin and appetite responses when in a state of energy balance have yet to be investigated. Individuals who regularly exercise but maintain a stable body weight, are typically in a prolonged state of energy balance rather than energy deficit, thus it would be interesting to observe whether similar changes in ghrelin and appetite regulation occur following exercise when the energy deficit is replaced and energy balance maintained.

The aims of the present study are two fold. 1) To determine the effects of exercise, with and without energy replacement, on appetite control, fasting and postprandial leptin and ghrelin concentrations. 2) To investigate whether leptin and ghrelin are related to subjective ratings of appetite following a single session of moderate intensity exercise.

5.2 Methods

Thirteen obese (BMI: 31.1 ± 3.0 kg.m-2, waist circumference: 105.6 ± 6.6 cm) middle-aged men (age: 39.9 ± 8.2 years) were recruited into this study. All subjects were healthy and non-smoking with no known diabetes or cardiovascular disease. All subjects completed a Bruce protocol exercise test as described in chapter 2.2 prior to participation to eliminate any cardiovascular contraindications to exercise.

5.2.1 Preliminary exercise and metabolic sessions

After an overnight fast of at least 12 hours, subjects attended the metabolic suite for measurement of resting metabolic rate and estimation of daily energy requirements (Chapter 2.4.1). Following 20 minutes of supine rest, a 25 minute expired air sample was collected with the mean of the final 20 minutes of data used for further analysis. Skinfold and circumference measurements as well as height and weight were made as described in Chapter 2.3.

Each subject completed two preliminary exercise tests prior to any experimental trials (Chapter 2.7). Briefly, a four-stage sub-maximal fitness test was used to estimate their $\dot{V}O_2$ max and calculate the speed and gradient required to elicit an intensity of 50% $\dot{V}O_2$ max. This speed and gradient was confirmed, and the associated net energy expenditure calculated, on a separate occasion using a 30-minute treadmill walk.

5.2.3 Experimental design

Each subject completed three separate two-day trials: control, exercise with energy deficit (energy-deficit) and exercise with energy replacement (energy-replacement). All trials were performed in random order at least one week apart. Day-one involved the control, energy-deficit or energy replacement intervention, which are explained in full in Chapter 3.2.5. Day-two comprised an 8.5 hour metabolic assessment. An overview of the two-day study design can be seen in **Figure 5.1**. Pre-trial controls were adhered to as described in Chapter 2.9.

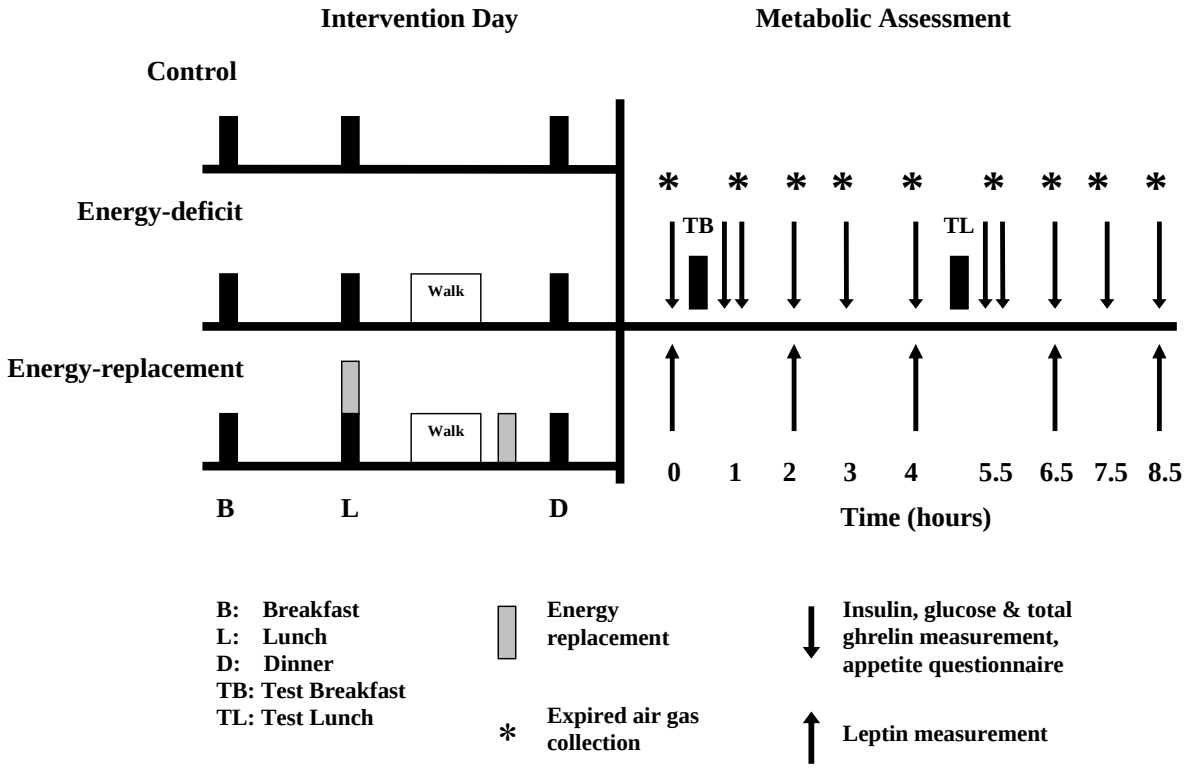


Figure 5.1 An overview of the two-day experimental protocol.

Day-two was exactly the same for each of the three intervention trials and an overview can be seen in Figure 5.1. Subjects attended the metabolic suite after an overnight fast of at least 12 hours, approximately 16 hours after completion of the treadmill walk. Fasting and postprandial blood samples were collected as described in Chapter 2.8.2.1 at 0, 0.5, 1, 2, 3, 4, 5, 5.5, 6.5, 7.5 and 8.5 hours. At exactly the same time points at which blood samples were collected, appetite questionnaires were also completed (Chapter 2.8.3). Expired air samples were collected (Chapter 2.4.1) during the 15 minutes preceding the 0, 1, 2, 3, 4, 5.5, 6.5, 7.5 and 8.5 hour time points. The test meal, described in Chapter 2.8.1, was provided on completion of fasting measurements and exactly the same meal was given again 4.5 hours later.

5.2.4 Blood analysis

Plasma leptin, total ghrelin, glucose and insulin concentrations were determined in the fasted state and at regular intervals during the postprandial period. A detailed description of these analyses can be seen in Chapter 2.10.

5.2.5 Data analysis

Energy expenditure and fat, carbohydrate and protein utilisation were calculated using indirect calorimetry as explained in Chapter 2.5. Energy expenditure, fat and carbohydrate oxidation were corrected for individual variations in body mass by dividing each subjects rate of energy expenditure, fat and carbohydrate oxidation by their body mass. HOMA was calculated to provide a marker of insulin resistance (Matthews *et al.*, 1985). The total AUC, calculated using the trapezium rule, divided by the duration of the observation period (8.5 hours) i.e. the time-averaged AUC and the incremental AUC, calculated as the increment in AUC over baseline concentrations, were used as a summary measures of the postprandial responses. It should be noted that, for variables where the values decrease from baseline postprandially, the calculated incremental AUC is negative and represents the area above the curve extending up to the baseline value, providing an index of postprandial suppression. The total AUC was used as a summary measure of the total energy expended and fat and carbohydrate oxidised during the metabolic assessment period.

Data were analysed using Statistica (version 6.0, StatSoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennsylvania). Prior to analysis, all data were tested for normality using the Anderson-Darling normality test and, if necessary, logarithmically transformed. Subsequently, leptin concentrations were transformed prior to statistical analysis. Appetite questionnaires were analysed as described in Chapter 2.8.3. Differences in the summary postprandial responses were analysed using repeated measures one-way ANOVA. Differences over time for the three trials were calculated using two-way ANOVA with repeated measures for trial and time. Post hoc Fisher least significant difference tests were used to identify where differences lay when main trial and or interaction effects were observed. Relationships between variables were assessed using Pearson-product correlations. Statistical significance was accepted at the $p < 0.05$ level and data are presented as mean ± SEM, unless otherwise stated.

5.3 Results

5.3.1 Responses during the treadmill walk

Subjects walked for 90.8 ± 3.6 minutes in both the energy-deficit and energy-replacement treadmill walks. The average walking speed and gradient of the incline were 5.5 ± 0.1 $km.h^{-1}$ and 3.4 ± 0.5%, respectively. Mean $\dot{V}O_2$ and heart rate were 19.1 ± 0.6 $ml.kg^{-1}.min^{-1}$ and 122 ± 3 $beat.min^{-1}$, respectively, for energy-deficit and 19.3 ± 0.6 $ml.kg^{-1}.min^{-1}$ and 123 ± 3 $beat.min^{-1}$, respectively, for energy-replacement. Net energy expenditure was 26.1 ± 0.4 $kJ.kg^{-1}$ body mass for energy-deficit and 24.2 ± 2.0 $kJ.kg^{-1}$ body mass for energy-replacement. Net fat and carbohydrate oxidation were 0.22 ± 0.02 $g.kg^{-1}$ body mass and 1.03 ± 0.05 $g.kg^{-1}$ body mass, respectively, during the energy-deficit walk and 0.20 ± 0.02 $g.kg^{-1}$ body mass and 1.08 ± 0.04 $g.kg^{-1}$ body mass, respectively, during the energy-replacement walk. The walks did not differ in any of the above variables. Both treadmill walks were tolerated well with subjects rating their effort as "fairly light" on the Borg Scale of 6-20 (Borg, 1973).

5.3.2 Metabolic Assessment: Responses in the fasted state

Table 5.1 shows the fasting values for the plasma and metabolic variables. Compared to control, fasting leptin concentrations were 17% ($p < 0.001$) and 9% ($p < 0.05$) lower in energy-deficit and energy-replacement, respectively. Fasting leptin was also 10% lower in energy-deficit compared to energy-replacement ($p< 0.01$). Fasting total ghrelin was 20% lower in energy-deficit compared with energy-replacement ($p < 0.01$). Fasting glucose and insulin concentrations and HOMA-IR did not differ significantly between trials ($p > 0.05$ for all). In the fasted state, the rate of fat oxidation was 33% higher in energy-deficit compared to control ($p < 0.01$) and 20% higher in energy-deficit compared to energy-replacement ($p < 0.05$). Fat oxidation was not different between control and energy-replacement ($p > 0.05$). The rate of carbohydrate oxidation was 27% lower in energy-deficit compared to control ($p < 0.05$). There were no significant differences in the rate of carbohydrate oxidation between energy-replacement and control ($p >0.05$) or between the two exercise trials ($p > 0.05$).

Table 5.1 Plasma and metabolic values measured in the fasted state

	Control	Energy-deficit	Energy-replacement
Leptin (ng.ml^{-1})	11.74 ± 2.54	9.71 ± 2.07[a,b]	10.69 ± 2.57[a]
Total ghrelin (ng.ml^{-1})	0.86 ± 0.09	0.76 ± 0.08[b]	0.91 ± 0.13
Glucose (mmol.l^{-1})	5.28 ± 0.13	5.19 ± 0.12	5.17 ± 0.14
Insulin (µU.l^{-1})	9.45 ± 1.08	9.05 ± 1.17	8.85 ± 1.2
HOMA-IR	2.25 ± 1.10	2.10 ± 1.03	2.05 ± 1.10
Energy expenditure (kJ.hr^{-1}.kg^{-1})	3.30 ± 0.07	3.35 ± 0.08	3.30 ± 0.09
Fat Oxidation (g.hr^{-1}.kg^{-1})	0.033 ± 0.003	0.044 ± 0.004[a,b]	0.035 ± 0.004
CHO Oxidation (g.hr^{-1}.kg^{-1})	0.077 ± 0.007	0.056 ± 0.008[a]	0.072 ± 0.010

N = 13. Values are mean ± SEM. [a]different to control ($p < 0.05$), [b]different to energy-replacement ($p < 0.05$). HOMA-IR: homeostasis model assessment of insulin resistance, CHO: carbohydrate.

Subjective ratings of appetite in the fasted state are shown in **Figure 5.2**. There were no significant differences in fasting ratings of hunger (control: 66.7 ± 7.2 mm, energy-deficit: 64.8 ± 6.1 mm, energy-replacement: 62.7 ± 4.9 mm), satiety (control: 31.9 ± 7.3 mm, energy-deficit: 32.5 ± 6.7 mm, energy-replacement: 30.5 ± 4.7 mm), fullness (control: 27.5 ± 7.2 mm, energy-deficit: 28.5 ± 6.1 mm, energy-replacement: 29.2 ± 5.0 mm), PFC (control: 72.1 ± 6.6 mm, energy-deficit: 69.8 ± 4.3 mm, energy-replacement: 73.7 ± 3.9 mm) or desire (control: 61.7 ± 8.0 mm, energy-deficit: 67.1 ± 6.2 mm, energy-replacement: 63.2 ± 5.1 mm) between the three trials ($p > 0.05$ for all).

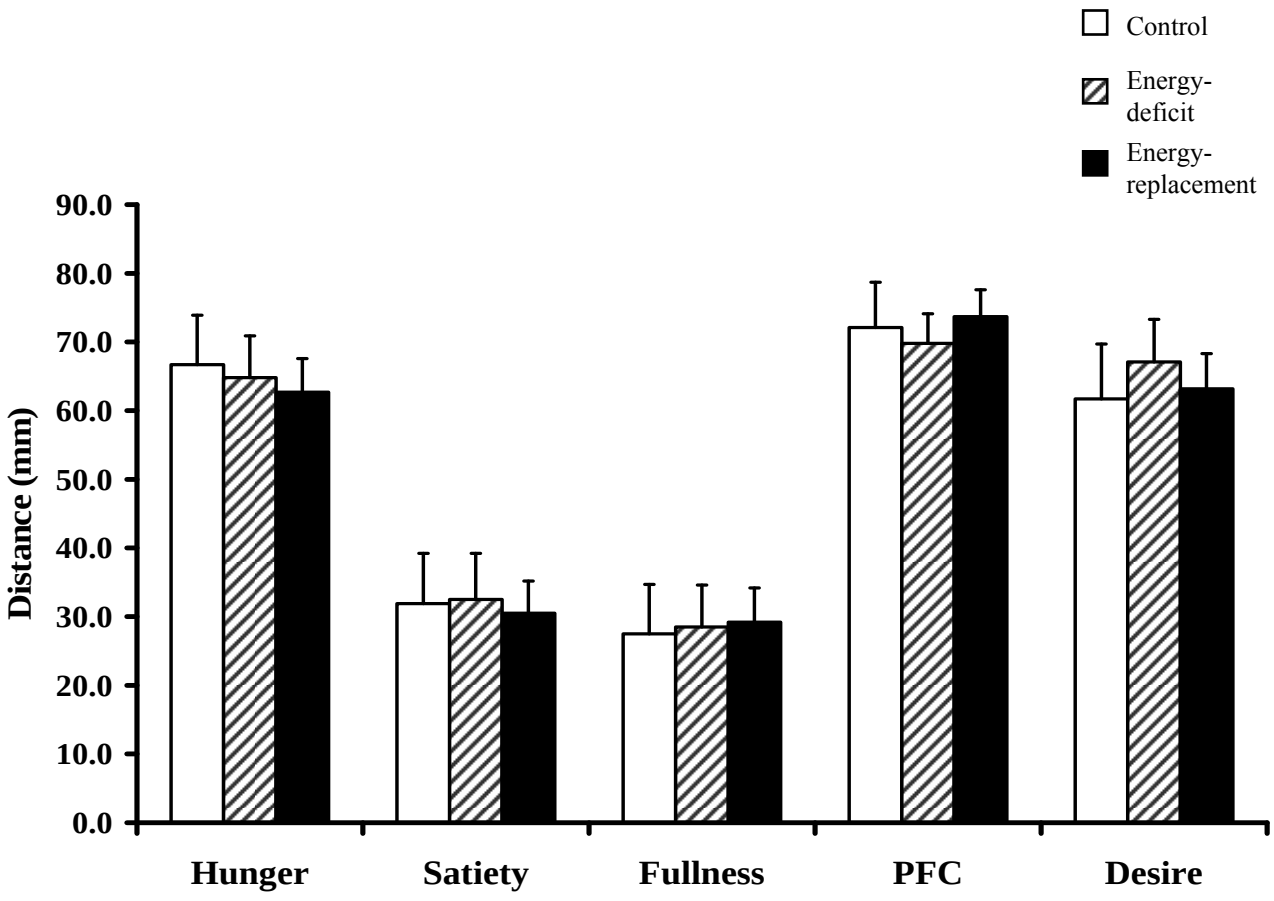


Figure 5.2 Mean subjective ratings of appetite in the fasted state.

5.3.3 Metabolic assessment: Responses in the postprandial state

Table 5.2 provides a summary of the postprandial leptin, total ghrelin, glucose and insulin responses. The postprandial leptin response during each trial is shown in **Figure 5.3** and the mean time-averaged leptin concentrations are presented in **Figure 5.4**. Compared to control, the postprandial leptin time-averaged AUC was 17% lower in energy-deficit ($p < 0.001$) and 7% lower in energy replacement ($p < 0.05$). There was no significant difference in the leptin response between the two exercise trials ($p > 0.05$). Postprandial total ghrelin responses did not differ significantly between the three trials ($p > 0.05$ for all, **Figure 5.5**). No significant differences were observed between trials in either the leptin or total ghrelin incremental AUC ($p > 0.05$ for all).

Table 5.2 Summary postprandial plasma and metabolic responses

	Control	Energy-deficit	Energy-replacement
Leptin ($ng.ml^{-1}$)			
Time-averaged AUC	10.98 ± 2.4	9.12 ± 1.91[a]	10.23 ± 2.25[a]
Incremental AUC	-0.77 ± 0.27	-0.58 ± 0.26	-0.46 ± 0.40
Total ghrelin ($ng.ml^{-1}$)			
Time-averaged AUC	0.79 ± 0.07	0.81 ± 0.07	0.78 ± 0.07
Incremental AUC	-0.06 ± 0.05	0.05 ± 0.05	-0.13 ± 0.07
Glucose ($mmol.l^{-1}$)			
Time-averaged AUC	5.62 ± 0.12	5.76 ± 0.12	5.77 ± 0.16
Incremental AUC	0.34 ± 0.13	0.57 ± 0.11[a]	0.60 ± 0.12[a]
Insulin ($\mu U.l^{-1}$)			
Time-averaged AUC	44.11 ± 5.00	36.00 ± 3.80[a,b]	39.60 ± 4.51[a]
Incremental AUC	34.62 ± 4.11	26.95 ± 3.16[a,b]	30.72 ± 3.61[a]

N = 13. Values are mean ± SEM. [a]different to control ($p < 0.05$), [b]different to energy-replacement ($p < 0.05$). AUC: area under the 8.5 hour concentration *vs*. time curve.

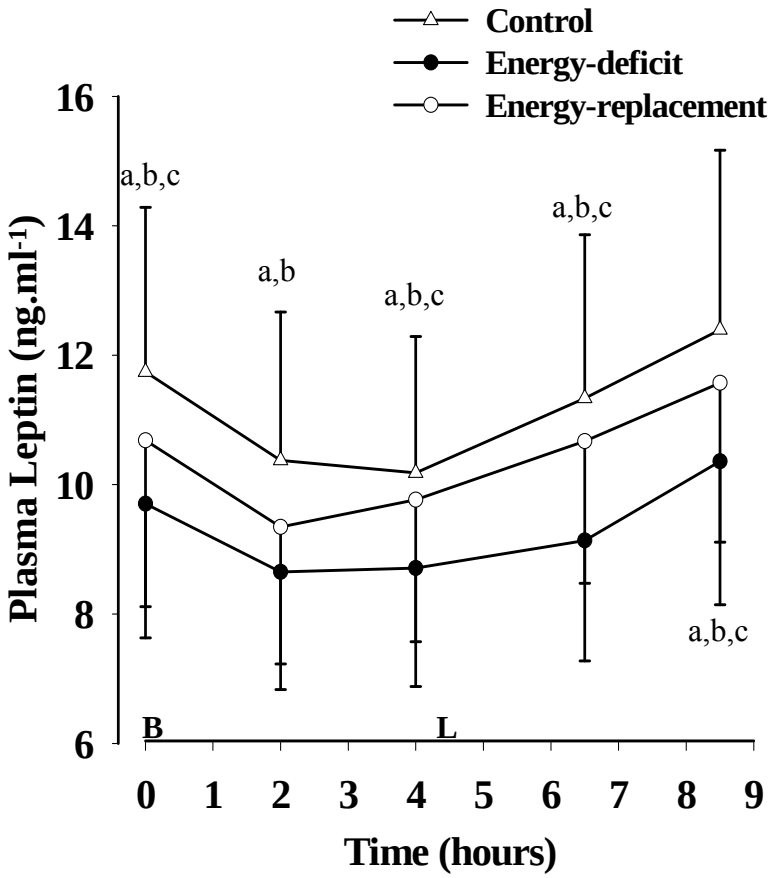


Figure 5.3 Postprandial leptin response for control (Δ), energy-deficit (●) and energy-replacement (o). B and L indicate the times at which the test breakfast (B) and test lunch (L) were provided. a: energy-deficit different to control ($p < 0.05$), b: energy-replacement different to control ($p < 0.05$), c: energy deficit different to energy-replacement ($p < 0.05$).

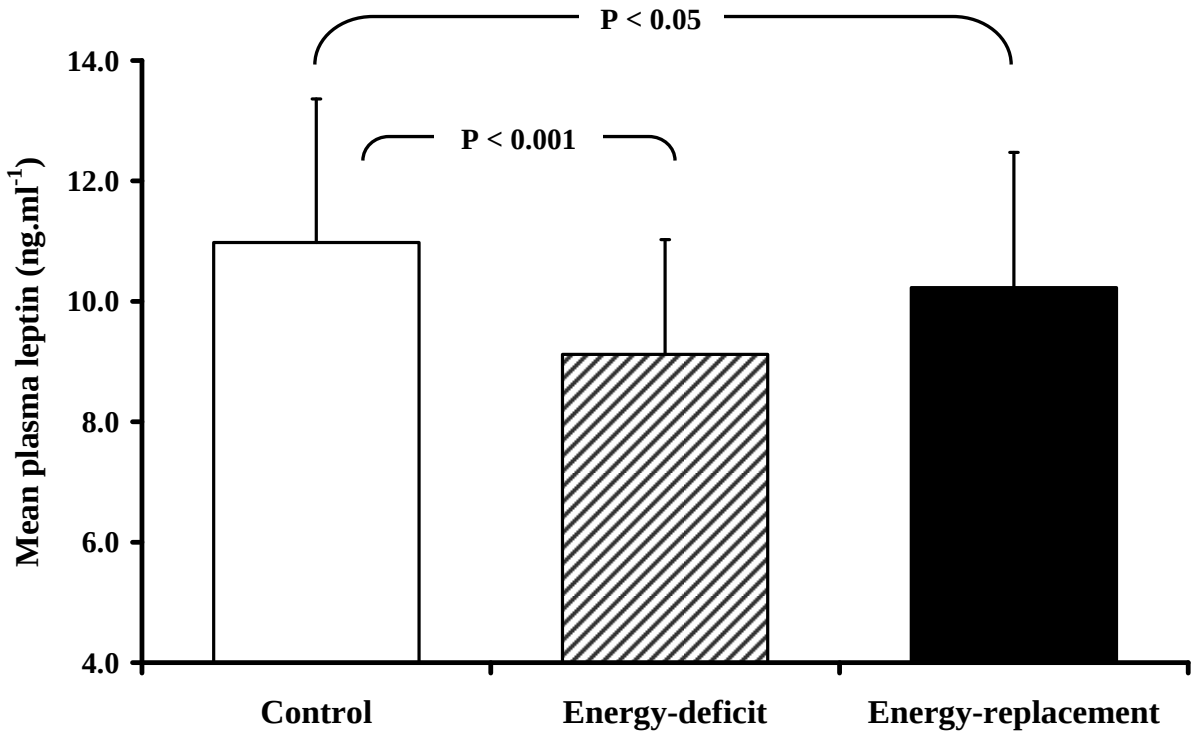


Figure 5.4 Time-averaged postprandial leptin response for control, energy-deficit and energy-replacement.

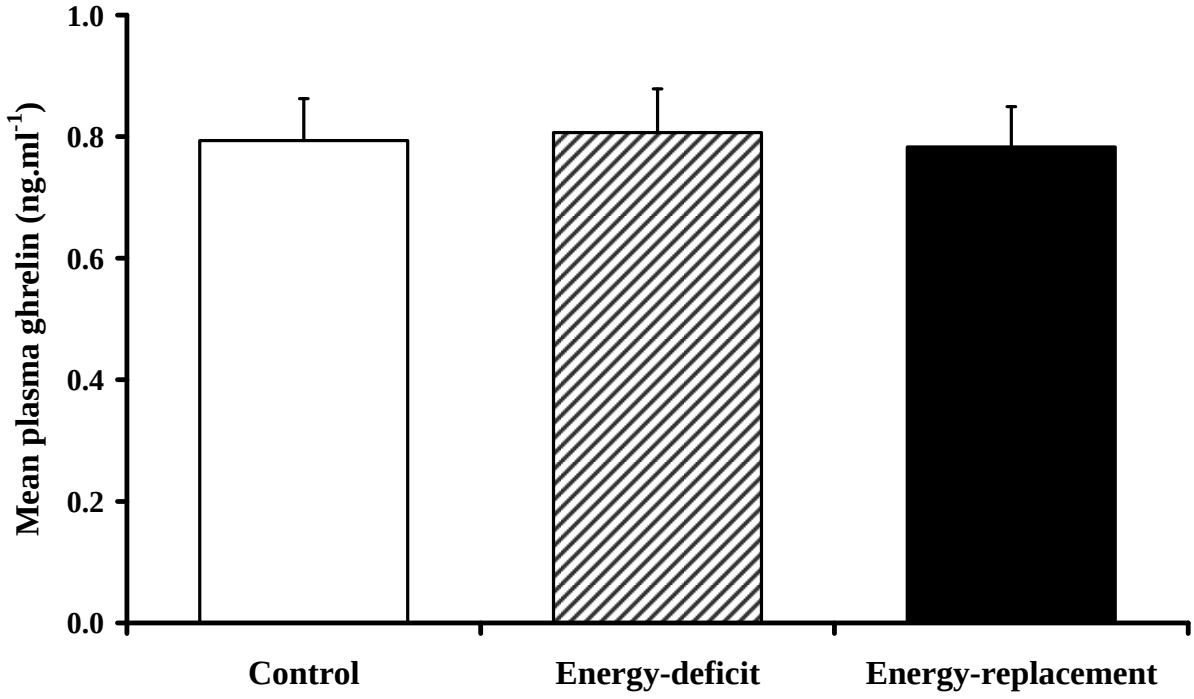


Figure 5.5 Time-averaged postprandial total ghrelin response for control, energy-deficit and energy-replacement.

Postprandial glucose concentrations did not differ between trials ($p > 0.05$), however the postprandial insulin time-averaged AUC was 18% lower and 10% lower in energy deficit ($p < 0.001$) and energy-replacement ($p < 0.01$), respectively, compared to control. The postprandial insulin time-averaged AUC was also 10% lower in energy-deficit compared to energy-replacement ($p < 0.05$) (readers are referred back to Figure 3.3 in chapter 3 which shows the postprandial glucose and insulin responses for each trial). When the changes in postprandial concentrations above baseline values were calculated, the glucose incremental AUC was 68% higher in energy-deficit ($p < 0.05$) and 76% higher in energy-replacement ($p < 0.05$) compared to control. The insulin incremental AUC was 22% and 11% lower in energy-deficit ($p < 0.001$) and energy-replacement ($p < 0.01$), respectively, compared to control. The insulin incremental AUC was also 14% lower in energy-deficit compared to energy-replacement ($p < 0.01$).

Energy expenditure during the postprandial metabolic observation period did not differ between trials (control: 31.54 ± 0.82 kJ.kg^{-1} body mass, energy-deficit: 31.82 ± 0.85 kJ.kg^{-1} body mass, energy-replacement: 31.58 ± 0.84 kJ.kg^{-1} body mass, $p > 0.05$ for all). **Figure 5.6** shows both postprandial fat and carbohydrate oxidation during the 8.5-hour metabolic assessment period. Over the 8.5-hour metabolic assessment, total fat oxidation in the control trial was 0.26 ± 0.01 g.kg^{-1} body mass. Total fat oxidation increased by 31% to 0.34 ± 0.02 g.kg^{-1} body mass in energy-deficit ($p < 0.001$) and by 15% to 0.30 ± 0.02 g.kg^{-1} body mass in energy-replacement ($p < 0.05$) compared to control. Fat oxidation was also 12% higher in energy-deficit compared to energy replacement ($p < 0.05$). Over the same 8.5 hour metabolic assessment period, total carbohydrate oxidation was 0.90 ± 0.06 g.kg^{-1} body mass in the control trial. Total carbohydrate oxidation decreased by 18% to 0.74 ± 0.05 g.kg^{-1} body mass in energy-deficit compared to control ($p < 0.001$). The 9% decrease in carbohydrate oxidation to 0.82 ± 0.05 g.kg^{-1} body mass in the energy-replacement trial compared to control showed a tendency to be different ($p = 0.058$) and a similar trend was observed for the 11% decrease in carbohydrate oxidation in energy-deficit compared to energy-replacement ($p = 0.056$).

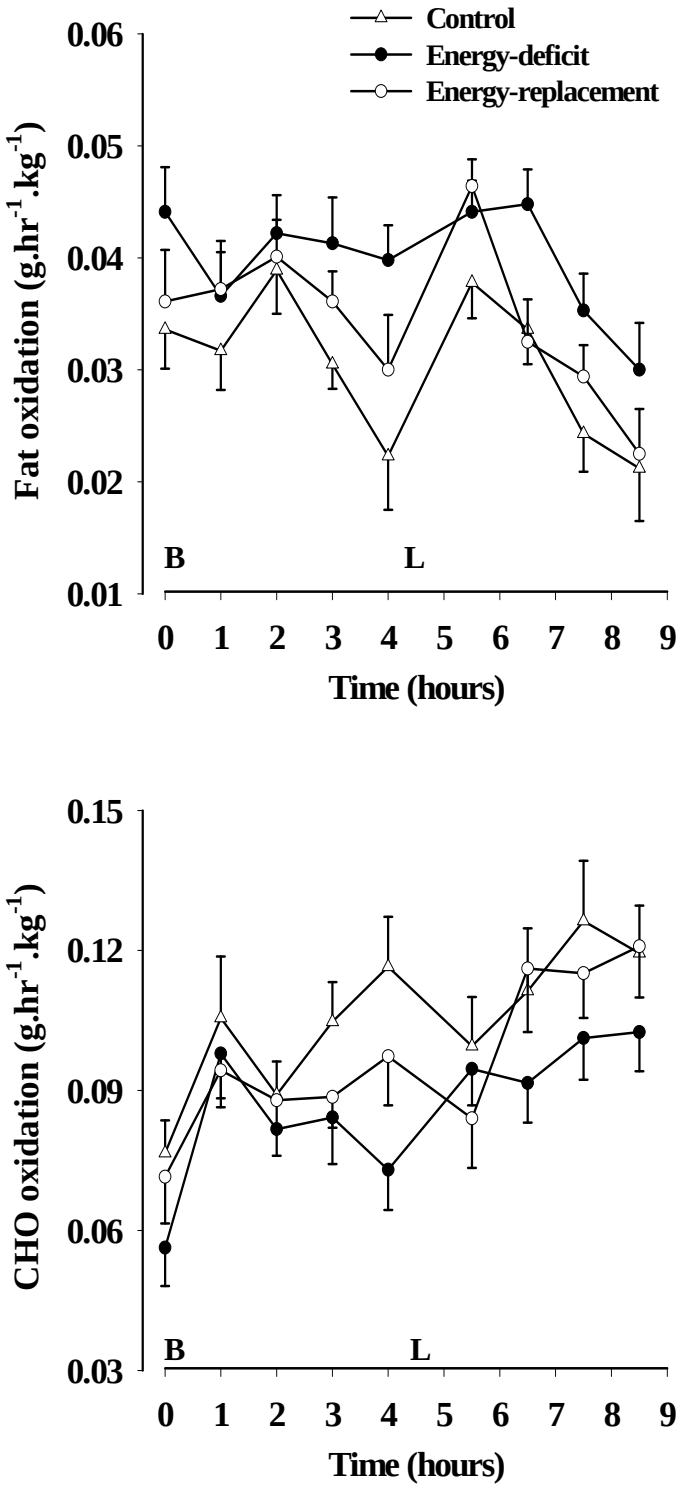


Figure 5.6 Fat oxidation (top) and carbohydrate oxidation (CHO, bottom) during the 8.5 hour metabolic assessment for control (Δ), energy-deficit (●) and energy-replacement (o). B and L indicate the times at which the test breakfast (B) and test lunch (L) were provided.

Mean postprandial appetite responses during the 8.5 hour observation period for each trial are shown in **Figure 5.7**. There were no significant differences between trials in subjective ratings of hunger (control: 46.2 ± 5.5 mm, energy-deficit: 46.8 ± 6.2 mm, energy-replacement: 47.6 ± 5.6 mm) satiety (control: 53.5 ± 5.7 mm, energy-deficit: 52.0 ± 5.9 mm, energy-replacement: 53.2 ± 5.6 mm), fullness (control: 50.8 ± 6.0 mm, energy-deficit: 50.7 ± 6.0 mm, energy-replacement: 50.4 ± 5.6 mm), PFC (control: 53.2 ± 6.2, energy-deficit: 54.7 ± 7.7 mm, energy-replacement: 55.7 ± 6.6 m) or desire (control: 48.2 ± 5.9 mm, energy-deficit: 48.3 ± 6.6 mm, energy-replacement: 48.9 ± 6.2 mm) ($p > 0.05$ for all).

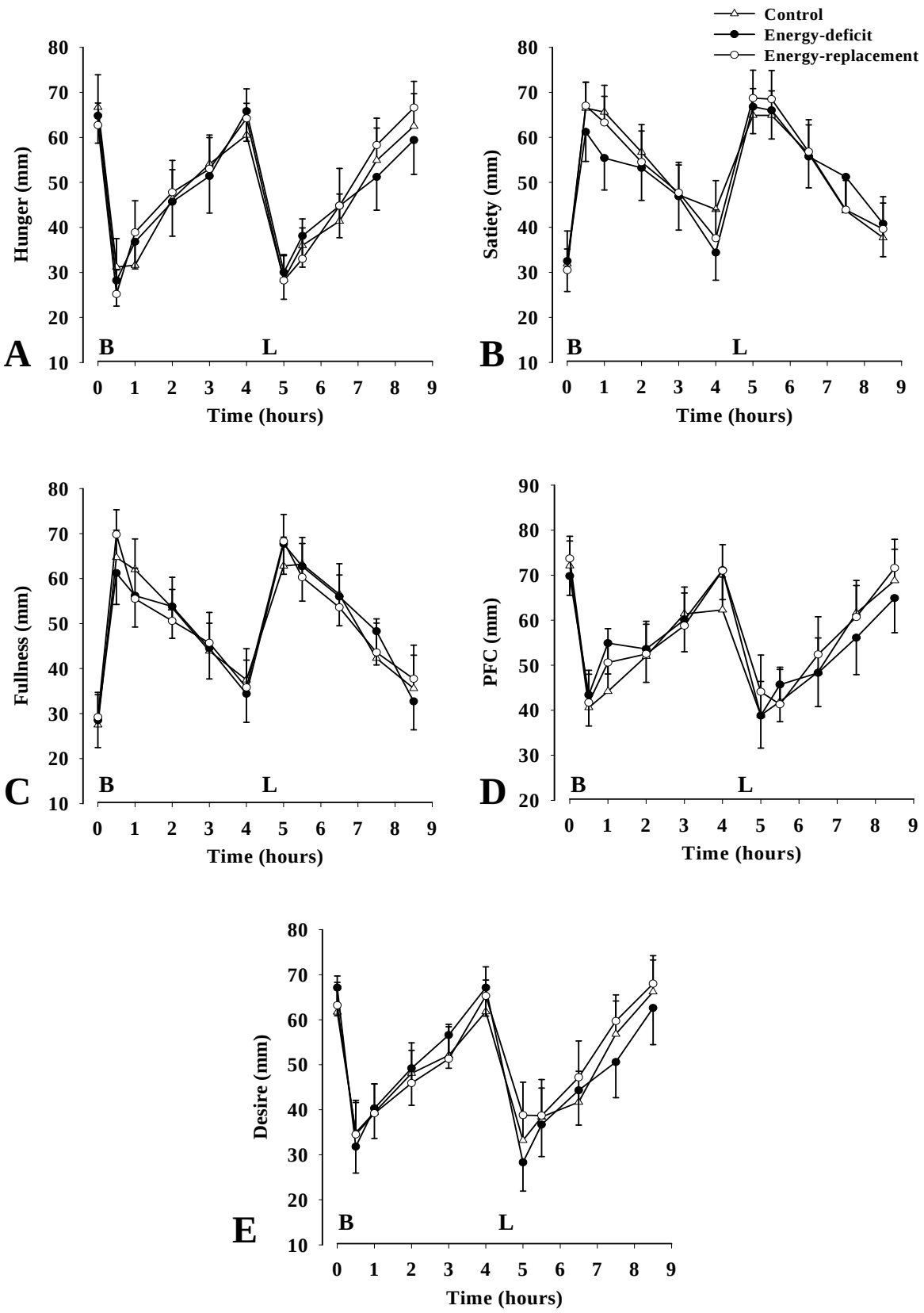


Figure 5.7 Hunger (A), satiety (B), fullness (C), prospective food consumption (PFC, D) and desire (E) ratings during control (Δ), energy-deficit (●) and energy-replacement (○). B and L indicate the times at which the test breakfast (B) and test lunch (L) were provided.

5.3.4 Relationships between variables

5.3.4.1 Leptin

Fasting leptin concentrations strongly correlated with the postprandial leptin time-averaged AUC in the control ($r = 0.99$, $p < 0.001$), energy-deficit ($r = 0.99$, $p < 0.001$) and energy-replacement ($r = 0.99$, $p < 0.001$) trials. Fasting leptin concentrations were related to BMI (control: $r = 0.71$, energy-deficit: $r = 0.66$, energy-replacement: $r = 0.70$, $p < 0.05$ for all) and waist circumference (control: $r = 0.66$, energy-deficit: $r = 0.73$, energy-replacement: $r = 0.67$, $p < 0.05$ for all). Postprandial leptin responses were also related to BMI (control: $r = 0.70$, energy-deficit: $r = 0.63$, energy-replacement: $r = 0.65$, $p < 0.05$ for all) and waist circumference (control: $r = 0.64$, energy-deficit: $r = 0.67$, energy-replacement: $r = 0.63$, $p < 0.05$ for all). When all trials were considered together, a significant correlation was observed between leptin and insulin concentrations in the fasting ($r = 0.49$, $p< 0.01$) and postprandial state ($r = 0.60$, $p < 0.001$) and also between leptin and HOMA-IR in the fasting ($r = 0.44$, $p <0.01$) and postprandial ($r = 0.42$, $p < 0.01$) state. No significant correlations were observed between leptin and any of the appetite ratings (hunger, satiety, fullness, PFC, desire) in either the fasting or postprandial states.

5.3.4.2 Total ghrelin

Fasting ghrelin concentrations strongly correlated with the postprandial ghrelin time-averaged AUC in the control ($r = 0.86$, $p < 0.001$), energy-deficit ($r = 0.81$, $p < 0.001$) and energy-replacement ($r = 0.93$, $p < 0.001$) trials. Neither fasting nor postprandial plasma ghrelin concentrations were correlated with BMI or waist circumference ($p > 0.05$ for both). Total ghrelin showed no correlation with plasma glucose or insulin concentrations, HOMA-IR, carbohydrate or fat oxidation whether measured in the fasting or postprandial state. When considered together, fasting ghrelin concentrations were related to hunger ($r = -0.475$, $p < 0.01$), satiety ($r = 0.49$, $p < 0.01$), fullness ($r = 0.50$, $p < 0.01$) and desire ($r = -0.47$, $p < 0.01$). Postprandial ghrelin concentrations were related to hunger ($r = -0.52$, $p < 0.01$), satiety ($r = 0.58$, $p < 0.001$), fullness ($r = 0.48$, $p < 0.01$), PFC ($r = -0.41$, $p < 0.01$) and desire ($r = -0.50$, $p < 0.01$).

5.3.5 Variables predicting the exercise-induced change in leptin

A significant correlation was observed between the exercise-induced change in fasting leptin and the exercise-induced change in postprandial leptin concentrations ($r = 0.90$, $p < 0.001$). Exercise-induced changes in the postprandial leptin time-averaged AUC were related to exercise-induced changes in postprandial glucose concentrations ($r = 0.48$, $p < 0.05$), as shown in **Figure 5.8**. Additionally, exercise-induced changes in the postprandial

leptin response were correlated with exercise-induced changes in both the carbohydrate oxidation total AUC ($r = 0.51$, $p < 0.05$) and the fat oxidation total AUC ($r = -0.44$, $p < 0.05$) as shown in **Figure 5.9**. Finally, a significant correlation was observed between the exercise-induced changes in postprandial leptin time-averaged AUCs and the changes in the postprandial ghrelin responses ($r = -0.46$, $p < 0.05$) (**Figure 5.10**).

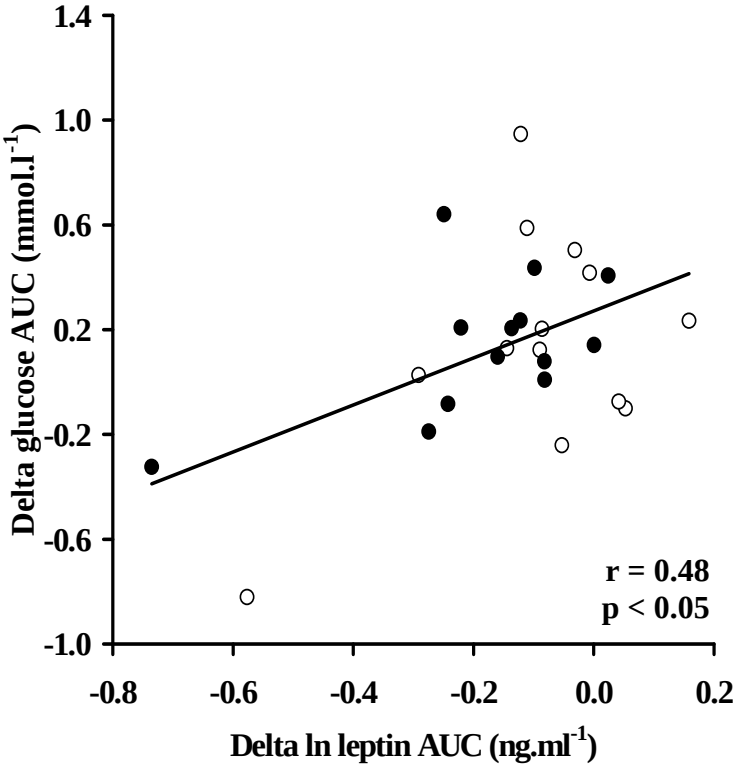
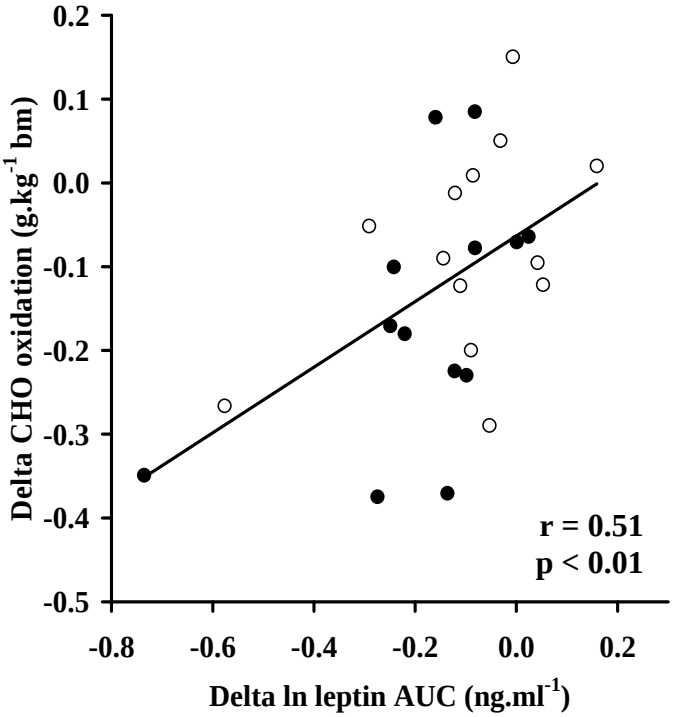


Figure 5.8 The relationship between exercise-induced changes in postprandial leptin and postprandial glucose concentrations (energy-deficit minus control: ●, energy-replacement minus control: o).



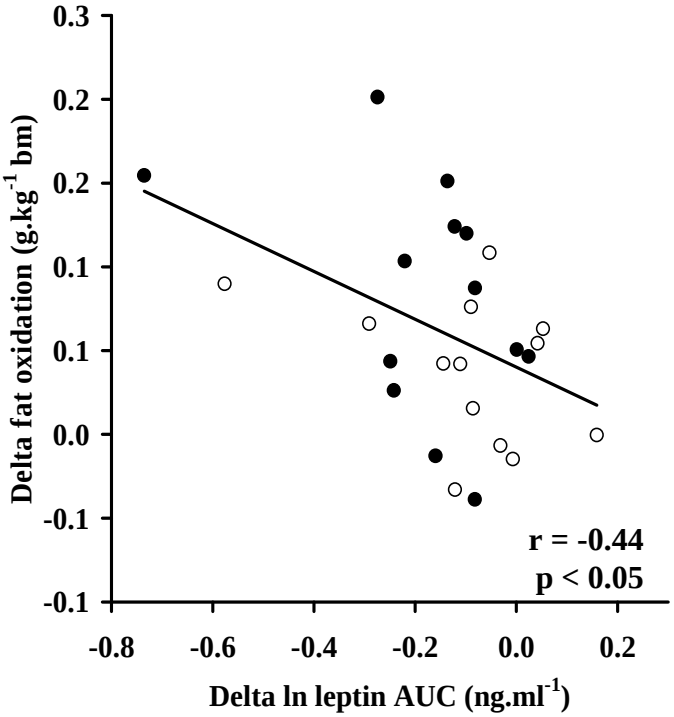


Figure 5.9 The relationship between exercise-induced changes in postprandial leptin and postprandial carbohydrate (CHO) oxidation (top) and exercise-induced changes in postprandial leptin and postprandial fat oxidation (bottom) (energy-deficit minus control: ●, energy-replacement minus control: o).

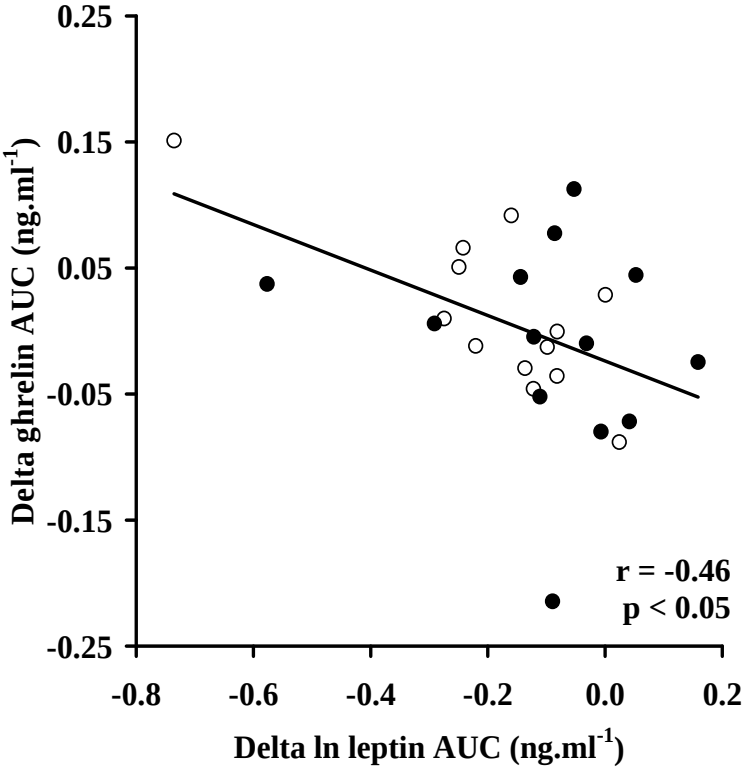


Figure 5.10 The relationship between exercise-induced changes in postprandial leptin and postprandial ghrelin concentrations (energy-deficit minus control: •, energy-replacement minus control: o).

5.4 Discussion

The aims of the present study were to investigate 1) The effect of exercise, with and without energy replacement, on appetite control and plasma leptin and total ghrelin concentrations and 2) To determine whether leptin and ghrelin concentrations were related to appetite ratings following acute exercise. Data from the present study show that a single session of moderate intensity exercise did not effect either plasma total ghrelin concentrations or subjective ratings of appetite, which included hunger, satiety, fullness, PFC and desire to eat. The same moderate intensity exercise session did, however, attenuate fasting and postprandial leptin concentrations, both with and without energy replacement. Furthermore, exercise-induced changes in postprandial leptin concentrations were, at least in part, explained by changes in postprandial glucose concentrations and energy substrate oxidation.

Evidence for the role of exercise in mediating subjective ratings of appetite is somewhat equivocal (Broom *et al.*, 2007;Hubert *et al.*, 1998;Imbeault *et al.*, 1997;Tsofliou *et al.*, 2003;Westerterp-Plantenga *et al.*, 1997a); the finding in this study of no significant effect

of exercise, either with or without energy replacement, on any measure of appetite, is consistent with the findings of Hubert *et al*. (Hubert *et al.*, 1998) and Imbeault *et al*. (Imbeault *et al.*, 1997). However, Westerterp-Plantenga *et al*. (Westerterp-Plantenga *et al.*, 1997a) and Broom *et al*. (Broom *et al.*, 2007) reported that exercise suppressed hunger whist Tsofliou *et al*. (Tsofliou *et al.*, 2003) reported increased satiety post-exercise. It is possible that by measuring appetite the day after the energy-deficit and energy-replacement exercise interventions, any immediate effect of exercise reported by others has been overlooked. The use of VAS appetite questionnaires with no direct measure of food intake should also be considered. The extent to which appetite questionnaires accurately reflect changes in food intake is unclear (Mattes *et al.*, 2005) thus the exercise interventions used in the present study could conceivably have altered food intake in the absence of any change in appetite. Earlier studies have, however, failed to show any effect of acute aerobic exercise in reducing energy intake (Hubert *et al.*, 1998;King *et al.*, 1996;King *et al.*, 1997a), thus it remains unclear whether the energy-deficit and energy-replacement trials will have had any effect on subsequent food intake. Additionally, VAS questionnaires provide subjective measures of appetite, which are likely regulated to some degree by learned behaviours (Herman & Polivy, 2005); exercise may be an inadequate stimulus to overcome such behaviour (Hubert *et al.*, 1998), thus subjective ratings of appetite will remain unchanged. Considering the potential limitations with the protocol applied in this study, future research is warranted to determine the immediate effects of exercise with energy replacement not only on appetite control but also food intake.

A role for ghrelin in the short-term regulation of appetite and energy balance is widely accepted (Klok *et al.*, 2007) although, consistent with the literature (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a), data from the present study showed no change in total ghrelin concentrations following a single session of exercise. Furthermore, exercise, even when the associated energy deficit was replaced and energy balance maintained, had no effect on total ghrelin concentrations. These findings add to the earlier research in a number of ways. Firstly, in addition to normal weight populations, it is shown here that exercise does not attenuate total ghrelin concentrations in obese middle-aged men. Furthermore, in contrast to earlier studies, plasma ghrelin was measured in the present study after a delay of approximately 16 hours. A potential criticism of studies investigating the immediate effect of exercise on plasma leptin was that their observation period was not long enough to take into account any delayed effect of exercise in altering the leptin response (Essig *et al.*, 2000;Landt *et al.*, 1997;Olive & Miller, 2001), a criticism that could also be extended to studies

investigating the effects of exercise on total ghrelin (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Martins *et al.*, 2007a). In obese men however, the current data suggest that there is not a delayed ghrelin response to exercise, at least within the ~ 24 hour post-exercise period. Finally, the exercise implemented in previous studies has varied in duration but none has been longer than one hour. It is possible that the associated energy expenditures and energy deficits were not sufficient to mediate any change in total ghrelin responses. The 90 minutes of exercise with an associated net energy expenditure of approximately 670 kcals (2.8 MJ) used in the present study did however, also fail to attenuate total ghrelin concentrations suggesting that if a threshold energy expenditure is needed for exercise to influence ghrelin, this is higher than the energy expenditure in the present study.

Similar to earlier studies (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a), the response of total ghrelin to exercise was measured in the present study. A recent and exciting development surrounds the finding that ghrelin comprises two individual components, acylated and de-acylated ghrelin (Hosoda *et al.*, 2006). Acylated ghrelin is suggested to contribute extensively to appetite control, whilst the role for de-acylated ghrelin appears minimal (Broom *et al.*, 2007;Mackelvie *et al.*, 2007). It seems that rather than attenuating total ghrelin, exercise may modulate the ratio of acylated to de-acylated ghrelin, a change that is unlikely to be observed in measurements of only total ghrelin. At present, research is limited, although a recent study has shown a single session of exercise to significantly lower acylated ghrelin concentrations (Broom *et al.*, 2007). Therefore exercise might still be a successful intervention for regulating appetite and food intake but via its effect on acylated and de-acylated ghrelin rather than total ghrelin *per se*.

The absence of any positive correlation between total ghrelin and hunger is consistent with data published by Burns *et al*. (Burns *et al.*, 2007) who found no correlation between hunger and ghrelin during the immediate post-exercise period. Rather unexpectedly, a negative correlation between ghrelin and hunger and conversely a positive correlation between ghrelin and satiety was observed, findings which were opposite of what was expected. It is difficult to explain why such a relationship may have occurred, although it is tempting to suggest that following exercise, the normal orexigenic properties of ghrelin might in some respect be suppressed or even reversed in an obese population. This could explain why obese individuals fail to show increased food intake after exercise compared to lean populations (King *et al.*, 1997b). The measurement of only total ghrelin rather than

acylated and de-acylated ghrelin may however limit these findings and those of Burns *et al*. (Burns *et al.*, 2007). It was recently shown that exercise-induced changes in acylated ghrelin were associated with changes in hunger measured during and for a short time after exercise (Broom *et al.*, 2007). There is however an obvious need for further investigation before any relationship between total ghrelin and or acylated ghrelin and appetite can be confirmed.

Similar to ghrelin, the role for exercise in modulating plasma leptin is unclear. A number of studies report no effect of exercise on plasma leptin (Hickey *et al.*, 1996a;Kyriazis *et al.*, 2007;Perusse *et al.*, 1997;Torjman *et al.*, 1999;Tsofliou *et al.*, 2003) whilst others suggest that the leptin response is lower after acute exercise (Aggel-Leijssen *et al.*, 1999;Duclos *et al.*, 1999;Essig *et al.*, 2000;Keller *et al.*, 2005;Tuominen *et al.*, 1997). A threshold of ~ 530 to 800 kcals has been suggested as the required energy expenditure to have any effect on leptin metabolism (Nindl *et al.*, 2002). Indeed, the net energy expended during the 90 minute treadmill walk, used in the present study, of 670 kcals (2.8 MJ) falls within this threshold and might explain the lower leptin response we observed after the energy-deficit trial. However plasma leptin concentrations were also lower when the energy deficit induced by exercise was replaced, suggesting that the energy deficit associated with this exercise did not mediate the leptin response *per se*. Essig *et al*. (Essig *et al.*, 2000) reported that an energy expenditure of either 800 kcals or 1500 kcals both attenuated fasting leptin concentrations by 30% and furthermore, 24 hour leptin profiles were significantly lower in a state of energy balance with exercise compared to a state of energy balance at rest (Aggel-Leijssen *et al.*, 1999). Data from this study and those of others (Aggel-Leijssen *et al.*, 1999;Essig *et al.*, 2000) suggest that it is exercise *per se* that mediates lower plasma leptin concentrations rather than the change in energy balance status, i.e. the energy deficit, which the exercise induces. Interestingly, whilst both the energy-deficit and energy-replacement trials significantly attenuated both the fasting and postprandial leptin responses, calculation of the incremental AUC revealed no significant difference in the leptin response between trials. This suggests that the lower postprandial leptin concentrations following the two exercise trials were most likely mediated by changes in the fasting leptin response rather than changes in the postprandial response.

Leptin may be modulated by changes in carbohydrate availability (Hilton & Loucks, 2000), a lower carbohydrate availability attenuating leptin concentrations (Jenkins *et al.*, 1997). In the present study, exercise-induced changes in postprandial leptin were significantly and positively correlated to exercise-induced changes in postprandial glucose

concentrations (r = 0.48); a smaller glucose response was associated with a lower leptin response. A similar relationship was also observed between exercise-induced changes in postprandial leptin and whole-body carbohydrate oxidation (r = 0.51). At first the correlation between leptin and carbohydrate oxidation might appear somewhat surprising as one would expect an increase in oxidation to reduce carbohydrate availability thus lowering leptin which would be reflected by an inverse relationship. However, carbohydrate metabolism is tightly regulated with increased carbohydrate availability inducing higher rates of carbohydrate oxidation (Schutz *et al.*, 1989). Therefore, the positive correlation between exercise-induced changes in carbohydrate oxidation and leptin are likely a reflection of the similar relationship observed between exercise-induced changes in glucose and leptin. An inverse relationship between exercise-induced changes in postprandial leptin and postprandial whole-body fat oxidation was also observed. However, although a role for fat oxidation and its subsequent availability in regulating the leptin response cannot be discounted, it is perhaps more likely that this is evidence of the reciprocal relationship between carbohydrate and fat oxidation that may have occurred in the absence of any change in energy expenditure i.e. at a constant energy expenditure, increases in carbohydrate oxidation are coupled with decreases in fat oxidation. At this point, readers should be made aware that in the correlations between leptin and glucose, carbohydrate oxidation and fat oxidation, there are two points that appear to lay outwith the majority of the data, and in all three comparisons these two points appear to be driving the correlation. The data were checked for normality prior to analysis and subsequently the leptin data was logarithmically transformed to ensure normal distribution. The relationships observed between leptin and carbohydrate are consistent with the literature, and although these data should be considered with caution until the findings can be confirmed in future studies, they may provide further insight into how leptin is mediated in the post exercise period.

Mechanisms mediating the exercise-induced lowering in plasma leptin concentrations have not been fully elucidated and research is ongoing. Stress related hormones such as cortisol and catecholamines (Duclos *et al.*, 1999) are implicated in the regulation of leptin and following exercise, the levels of such hormones increase (Sliwowski *et al.*, 2001), which may explain any immediate and short-term changes in plasma leptin. However, levels of these hormones return to pre-exercise values relatively quickly (within two hours) (Sliwowski *et al.*, 2001) and as such are unlikely to explain the changes observed in plasma leptin some 16 hours after exercise. It is perhaps more likely that changes in plasma leptin are regulated by carbohydrate (glucose) availability via the hexosamine

biosynthetic pathway, a nutrient sensing pathway (Hulver & Houmard, 2003) and the end product of which, UDP-*N*-acetylglucosamine, stimulates leptin secretion (Considine *et al.*, 2000). Lower fasting glucose concentrations coupled with lower fasting and postprandial insulin concentrations may have restricted glucose uptake into adipocytes and along the pathway (Hulver & Houmard, 2003), thus attenuating plasma leptin concentrations. This hypothesis is however purely speculative and future research will hopefully further elucidate the role for glucose in the regulation of leptin metabolism.

The relationship between insulin and leptin is well documented and a positive correlation observed in the present study between both fasting and postprandial plasma leptin and insulin concentrations is consistent with others (Koutsari *et al.*, 2003;Okazaki *et al.*, 1999;Tuominen *et al.*, 1997). Additionally, and again similar to others, a positive relationship between HOMA-IR and leptin was observed (Franks *et al.*, 2007;Leyva *et al.*, 1998). Together these findings appear to provide clear evidence of a role for insulin in regulating leptin metabolism. There have, however, been questions raised as to whether it is the action of insulin *per se* that mediates leptin or whether it is the effect of insulin on glucose uptake and availability that determines the leptin response (Wellhoener *et al.*, 2000). In the present study, although no correlation between glucose and leptin concentrations were found, a correlation between the exercise-induced changes in glucose and leptin was reported. Furthermore, there was no significant relationship between exercise-induced changes in insulin and leptin and this might suggest that following a single session of exercise, glucose rather than insulin may mediate postprandial leptin concentrations.

Although traditionally a long term regulator of energy balance (Jequier, 2002;Klok *et al.*, 2007), the secretion of gastric leptin and its subsequent interaction with other appetite hormones, might provide leptin with a role in the short term regulation of appetite and energy balance (Klok *et al.*, 2007). Surprisingly, there is limited research investigating the effects of exercise-induced changes in leptin in relation to appetite and satiety ratings. Interestingly, Tsofliou *et al*. (Tsofliou *et al.*, 2003) reported that at rest and following a snack meal leptin was not related to any measure of appetite, however, after exercise leptin was correlated with satiety, hunger, fullness, PFC and desire to eat, suggesting that exercise improved the coupling between leptin and appetite in a way that was not evident after food intake. Unfortunately, data from this study fail to support any association between leptin and appetite ratings either at rest or after exercise. However, appetite and leptin were measured the day following exercise which may have overlooked an immediate

effect of exercise like that observed in Tsofliou's study. A gender effect could also explain the different results. The present study recruited obese men whereas Tsofliou *et al*. (Tsofliou *et al.*, 2003) studied obese women; appetite responses to exercise may differ between sexes (King *et al.*, 1997b). An interesting finding of the present study is the inverse association observed between exercise-induced changes in plasma leptin and plasma ghrelin concentrations. There is evidence that plasma leptin and ghrelin are negatively correlated (Halaas *et al.*, 1995;Tschop *et al.*, 2001) and leptin may mediate short term energy balance via an inhibitory effect on plasma ghrelin (Yildiz *et al.*, 2004), although findings are by no means equivocal (Klok *et al.*, 2007). To the best of the authors' knowledge, there are no other studies showing an association between exercise-induced changes in postprandial leptin and ghrelin concentrations and currently the mechanism relating the two hormones is not clear. In rodent models leptin has been shown to be an upstream regulator of ghrelin (Nakazato *et al.*, 2001), however this requires validation in human models. Despite the literature being inconclusive, these findings do suggest that as well as a long term effect, leptin may also be important for the short term regulation of energy balance, possibly via its interaction with other hormones such as ghrelin.

A strength of the present study is the measurement of leptin in response to exercise after a delay of ~16 hours and over a prolonged metabolic observation period. Many studies investigating the effects of exercise on leptin concentrations have used a single measurement of leptin taken immediately after exercise (Hickey *et al.*, 1996a;Perusse *et al.*, 1997;Racette *et al.*, 1997) or after a short recovery period (Tsofliou *et al.*, 2003). However, leptin appears to respond to exercise after a delay of upto 48 hours (Essig *et al.*, 2000;Olive & Miller, 2001), thus it is likely that studies showing no change in leptin after exercise have in fact simply failed to observe any changes due to the timing of leptin measurements. Measuring leptin the day after exercise increased the potential for observing and investigating any exercise-induced changes in leptin. Furthermore, plasma leptin was measured in the fasting state and in response to test meals of a mixed dietary composition. Using a study design like this provides insight into how leptin responds to exercise in the more typical and "real life" setting of consuming multiple meals with a fat and carbohydrate content reflective of the typical Scottish diet. The long delay between exercise and metabolic assessment may, however, also be a potential limitation of this study. It remains unknown how exercise, with and without energy replacement, effects leptin, ghrelin and appetite regulation immediately after exercise and this is a concern that needs to be addressed in future research.

Findings from the present study have demonstrated that total ghrelin and subjective ratings of appetite were not affected by prior exercise and that changes in appetite and ghrelin are therefore unlikely to contribute to the enhanced weight maintenance which occurs with regular exercise (Wareham *et al.*, 2005). In contrast, fasting and postprandial leptin concentrations were significantly attenuated following exercise, both with and without energy replacement, suggesting that the exercise-induced energy deficit *per se* does not determine the lower leptin response. It is likely that the postprandial leptin response is in part mediated by changes in energy substrate availability, in particular glucose. Finally, as well as long term regulation, exercise-induced changes in postprandial leptin may also be implicated in the short term regulation of energy balance via its interaction with and possible inhibition of plasma ghrelin.

CHAPTER 6
ENERGY BALANCE WITH HIGH AND LOW ENERGY TURNOVER: EFFECTS ON POSTPRANDIAL METABOLISM

6.1 Introduction

Metabolic perturbations occurring during the postprandial state contribute to the development and progression of atherosclerosis (Zilversmit, 1979). Humans typically spend a majority of the day in the postprandial state, and therefore interventions which alter postprandial metabolism will have important implications for the future prevention and management of cardio-metabolic diseases.

A single session of moderate intensity exercise is known to favourably alter postprandial metabolism. The day following acute exercise, postprandial lipaemia and insulinaemia are attenuated (Gill *et al.*, 2002b) and fat oxidation is also reported to be increased for at least 24 hours post exercise (Hansen *et al.*, 2005). The magnitude of these effects, especially with regard to the exercise-induced lowering of postprandial triglyceride (TG) concentrations, is related to the energy expended during the exercise session rather than to the intensity or duration of the exercise *per se* (Tsetsonis, 1996). Generally however, studies have not considered whether changes in postprandial metabolism are mediated by an exercise-induced energy deficit or by exercise alone. One study comparing the effects of exercise or dietary-induced energy deficits of similar magnitude on postprandial TG concentrations, showed greater attenuation in TG following exercise compared to dietary restriction (Gill & Hardman, 2000). This suggested that either the effects of exercise on postprandial metabolism were independent of the associated energy deficit or that the energy deficit was important but dietary and exercise-induced energy deficits had different metabolic effects. To investigate this hypothesis the effects of exercise, with and without energy replacement and therefore energy deficit, on postprandial metabolism were compared, as described in Chapter 3. An energy-deficit was needed to elicit an exercise-induced reduction in TG concentrations, however, postprandial insulinaemia was reduced and whole body fat oxidation increased even with energy replacement, although retaining the energy-deficit did augment such effects. Interestingly, peripheral pulse wave velocity (PWV) was attenuated by exercise independently of the associated energy deficit (Chapter 3), although the effect on central PWV was not measured and requires further investigation.

The findings from Chapter 3 suggest that a state of energy balance with high energy turnover i.e. energy balance coupled with exercise, is metabolically different to a state of energy balance with low energy turnover i.e. energy balance coupled with rest; postprandial insulin concentrations were significantly attenuated, whole body fat oxidation was elevated and peripheral PWV was lower the day following exercise with energy replacement compared to the control trial. The experimental protocol implemented in Chapter 3 however included a 16 hour delay between the end of exercise and the start of metabolic assessment. Consequently, it is not possible to comment on the immediate effects of energy balance with high energy turnover on postprandial metabolism. An immediate effect of acute exercise on postprandial lipaemia (Katsanos & Moffatt, 2004;Zhang *et al.*, 1998), insulinaemia (Englert *et al.*, 2006;Mikines *et al.*, 1988) and energy substrate utilisation (Saris & Schrauwen, 2004;Schneiter *et al.*, 1995;Votruba *et al.*, 2002) has already been reported, but, to the authors' knowledge, no study has investigated the immediate effect of acute exercise during a state of energy balance. Investigating the short-term changes following exercise in energy balance, might provide insight into how postprandial metabolism was mediated in a state of energy balance with high energy turnover on the day following exercise.

Therefore, the present study was designed to compare how a state of energy balance with high energy turnover i.e. energy balance with exercise, differs from a state of energy balance with low energy turnover i.e. energy balance with rest, with respect to postprandial metabolism and arterial stiffness.

6.2 Methods

Thirteen pre-menopausal women were recruited into this study. Their physical characteristics are shown in **Table 6.1**. All subjects were apparently healthy, non-smoking and with no known cardiovascular disease or diabetes. None was taking any medication thought to interfere with lipid or energy substrate metabolism.

Table 6.1 Physical characteristics of subjects.

Age (years)	32 ± 8
Body Mass Index (kg.m^{-2})	27.5 ± 1.1
Waist Circumference (cm)	87.1 ± 6.2
Systolic Blood Pressure (mmHg)	118 ± 13
Diastolic Blood Pressure (mmHg)	72 ± 8
Maximal oxygen consumption (ml.kg^{-1}.min^{-1})	34.7 ± 5.8

N = 13, values are mean ± SD.

6.2.1 Preliminary sessions

Each subject attended the metabolic suite having fasted overnight for at least 12 hours. After 20 minutes of rest lying on the couch, a 25-minute expired air sample was collected and their resting metabolic rate was calculated (Chapter 2.4.1). Daily energy requirements were calculated on an individual basis using a PAL of 1.2 (Chapter 2.4.1.1). Anthropometric measurements were made for all subjects as described in Chapter 2.3.

Each subject completed a four-stage sub-maximal fitness test (Chapter 2.7.1) to estimate their maximal oxygen consumption ($\dot{V}O_2$ max) and calculate the speed and gradient that was required to elicit an intensity of 50% $\dot{V}O_2$ max, the intensity that was used for the exercise in the high energy turnover trial.

6.2.2 Experimental design

Each subject completed two separate one-day trials; energy balance with low energy turnover (low energy turnover) and energy balance with high energy turnover (high energy turnover). Trials were performed in a randomised design and completed approximately four weeks apart to ensure that subjects were monitored in the same phase of their menstrual cycle for both trials. An overview of the study protocol is shown in **Figure 6.1**. For three days prior to each experimental trial, subjects were instructed to avoid alcohol and all planned exercise. Subjects were also asked to weigh and record all of the food and drink they consumed during the three days before the first trial and this was replicated during the three days leading up to the second trial (Chapter 2.9).

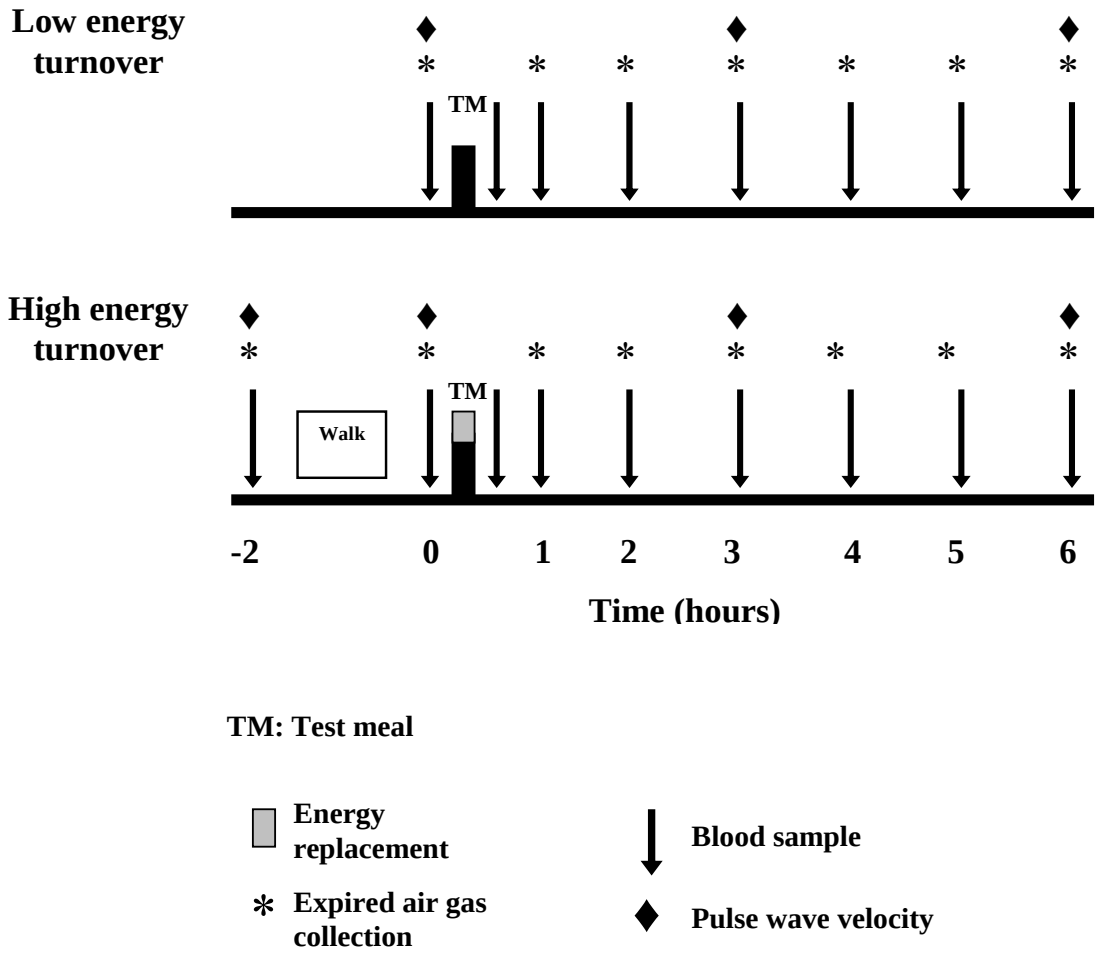


Figure 6.1 An overview of the experimental study design

6.2.3 Low energy turnover trial

Subjects attended the metabolic suite at 10:00 am having fasted from 8:00 pm the evening before. Fasting expired air measurements and blood samples were made as described in Chapters 2.4.1 and 2.8.2.1, respectively. Fasting femoral and radial PWV measurements were also made as described in Chapter 2.8.2.2. The test meal (Chapter 2.8.1) was provided on completion of all fasting measurements. During the postprandial metabolic assessment period, blood samples were collected at 0.5, 1, 2, 3, 4, 5 and 6 hours after the test meal. Expired air measurements were taken during the 15 minutes prior to the 1, 2, 3, 4, 5 and 6 hour blood samples and PWV was measured after the blood samples were collected at 3 and 6 hours (Figure 6.1).

6.2.4 High energy turnover trial

Subjects attended the metabolic suite at 8:00 am having fasted from 8:00 pm the night before. Fasting expired air, femoral and radial PWV measurement and blood samples were collected. On completion of these fasting measurements, subjects performed a 60-minute

treadmill walk at 50% $\dot{V}O_2$ max: Following five minutes rest, a resting five-minute expired air sample was collected (Chapter 2.4.2) and heart rate and RPE were recorded (Chapter 2.6). The treadmill walk was started at the speed and gradient, previously determined during the fitness test, to elicit an intensity of 50% $\dot{V}O_2$ max. Two minute expired air samples were collected at 13-15, 28-30, 43-45 and 58-60 minutes. $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated to ensure the correct exercise intensity was maintained. If the workload was too high or too low the gradient of the treadmill was adjusted accordingly. On completion of the walk, ten minutes of post-exercise recovery measurements were made. For the initial two minutes of recovery, the treadmill speed was lowered to 3.0 $km.h^{-1}$ and the gradient returned to 0%. After two minutes the treadmill was stopped and the subject was seated whilst remaining on the treadmill. During these 10 minutes, continual expired air samples were collected from 0-2, 2-5 and 5-10 minutes. Throughout the entire exercise intervention heart rate and RPE were measured during the final minute of each expired air collection. Net energy expenditure during the walk and recovery period were calculated using indirect calorimetry (Chapter 2.5).

On completion of the walk, subjects were allowed to shower and change, and fasting post-exercise expired air, femoral and radial PWV measurements and blood samples were repeated (Figure 6.1). After the post-exercise measurements, the same test meal as the one used in the low energy turnover trial was provided, but this time the energy content was increased to replace 110% of the net energy expended during the trial walk (Chapter 2.8.1). Postprandial blood samples, expired air and PWV measurements were collected in exactly the same way as described above for the low energy turnover trial and as shown in Figure 6.1.

6.2.5 Blood Analysis

In both trials, total cholesterol, LDL and HDL cholesterol were determined in the fasted state only. Plasma TG, NEFA, 3-OHB, glucose and insulin were determined in the fasting state, post-exercise (the high energy turnover trial only) and at regular intervals throughout the six hour postprandial observation period. For a more detailed description of these analyses refer to Chapter 2.10.

6.2.6 Data analysis

Energy expenditure and energy substrate utilisation, corrected for protein oxidation, were calculated using indirect calorimetry (Chapter 2.5). The total AUC, calculated using the

trapezium rule, divided by the duration of the observation period (six hours) i.e. the time-averaged AUC and also the incremental AUC, calculated as the increment in AUC over baseline concentrations were used as summary measures of the postprandial responses.

Data were analysed using Statistica (version 6.0, Statsoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennyslyvania). All data were tested for normality prior to any further analysis and no transformations were required. Differences between trials for fasting and post-exercise values and summary responses were analysed using paired samples t-tests. Differences over time for the two trials were calculated using two-way ANOVA with repeated measures for trial and time. Post hoc Fisher least significant difference tests were used to identify where differences lay when main trial and or interaction effects were observed. Relationships between variables were assessed using Pearson product-moment correlations. Statistical significance was accepted at the $p < 0.05$ level and data are presented as mean ± SEM unless otherwise stated.

6.3 Results

6.3.1 Responses during the treadmill walk

Subjects walked for one hour at a speed of 5.5 ± 0.1 $km.h^{-1}$ up a gradient of 2.4 ± 0.6%. All subjects completed the walk without difficulty, rating the exercise as 'light' (level 11.1 ± 0.3) on the Borg scale of 6-20 (Borg, 1973). Mean $\dot{V}O_2$ during the walk was 16.6 ± 0.7 $ml.kg^{-1}.min^{-1}$ and mean heart rate was 123 ± 2 $beats.minute^{-1}$. Net energy expenditure of the walk was 1.18 ± 0.07 MJ. Net fat and carbohydrate oxidation during the walk were 22.3 ± 1.4 g and 18.1 ± 3.3 g, respectively.

6.3.2 Plasma and metabolic variables in the fasted state

A summary of fasting plasma and metabolic variables for both trials is given in **Table 6.2**. A single fasting measurement was used in the low energy turnover trial and a fasting and post-exercise measurement in the high-energy turnover trial. Comparison of fasting measurements revealed no significant differences between trials in any of the variables except for TG concentrations, which were 17% higher in the high energy turnover trial than the low energy turnover trial ($p < 0.05$). Comparison of the low energy turnover trial fasting and high energy turnover trial post-exercise measurements revealed no significant differences in any variable other than plasma NEFA concentrations which were 34% higher in the high energy turnover trial ($p = 0.05$). When comparing fasting and post-exercise measurements within the high energy turnover trial, post-exercise TG concentrations were 13% lower ($p < 0.001$) whilst NEFA and 3-OHB concentrations were

36% ($p < 0.01$) and 189% ($p < 0.001$) higher, respectively, compared to fasting concentrations. Glucose concentrations did not change post-exercise, however insulin concentrations were 25% lower post-exercise compared to fasting ($p < 0.05$). Energy expenditure did not change post-exercise although there were changes in substrate utilisation. Fat oxidation was 23% higher post-exercise ($p < 0.01$) and carbohydrate oxidation was 45% lower post-exercise ($p < 0.05$) compared to fasting. Neither systolic nor diastolic blood pressure changed post-exercise compared to fasting. No significant change was observed in post-exercise femoral PWV but post-exercise radial PWV was 5% lower ($p < 0.05$) compared to fasting measurements.

Table 6.2 Plasma and metabolic values in the fasted state

	Low energy turnover: fasting	**High energy turnover: fasting**	**High energy turnover: post-exercise**
Total cholesterol ($mmol.l^{-1}$)	4.28 ± 0.18	4.36 ± 0.19	–
LDL cholesterol ($mmol.l^{-1}$)	2.48 ± 0.16	0.54 ± 0.18	–
HDL cholesterol ($mmol.l^{-1}$)	1.48 ± 0.08	1.45 ± 0.07	–
Triglyceride ($mmol.l^{-1}$)	0.70 ± 0.05	0.82 ± 0.09[a]	0.71 ± 0.08[b]
NEFA ($mmol.l^{-1}$)	0.59 ± 0.05	0.58 ± 0.05	0.79 ± 0.04[a,b]
3-hydroxybutyrate ($\mu mol.l^{-1}$)	271.9 ± 94.9	142.4 ± 42.2	409.8 ± 87.9[b]
Glucose ($mmol.l^{-1}$)	4.89 ± 0.05	4.99 ± 0.13	4.81 ± 0.08
Insulin ($mU.l^{-1}$)	3.82 ± 0.42	4.36 ± 0.52	3.29 ± 0.48[b]
Non-protein RQ	0.79 ± 0.02	0.80 ± 0.02	0.80 ± 0.01[b]
Energy Expenditure ($kJ.hr^{-1}$)	251.3 ± 6.5	251.5 ± 6.3	253.47 ± 6.61
Fat Oxidation ($g.hr^{-1}$)	3.73 ± 0.37	3.45 ± 0.32	4.25 ± 0.26[b]
CHO Oxidation ($g.hr^{-1}$)	3.12 ± 0.68	3.79 ± 0.68	2.09 ± 0.65[b]
Systolic BP (mmHg)	109 ± 3	109 ± 4	113 ± 4
Diastolic BP (mmHg)	66 ± 1	69 ± 2	67 ± 2
Femoral PWV ($m.s^{-1}$)	6.87 ± 0.25	6.87 ± 0.19	6.94 ± 0.21
Radial PWV ($m.s^{-1}$)	8.81 ± 0.23	9.24 ± 0.33	8.75 ± 0.20[b]

N = 13, values are mean ± SEM. [a]different to low energy turnover: fasting ($p < 0.05$), [b]different to high energy turnover: fasting ($p < 0.05$). NEFA: non-esterified fatty acids, LDL: low density lipoprotein, HDL: high density lipoprotein, RQ: respiratory quotient, CHO: carbohydrate, BP: blood pressure, PWV: pulse wave velocity.

6.3.3 Postprandial plasma and metabolic variables

Summary measures of the postprandial values are given in **Table 6.3.** Postprandial TG, NEFA and 3-OHB responses and glucose and insulin responses are shown in **Figures 6.2** and **6.3**, respectively. There were no significant differences in the time-averaged postprandial TG or 3-OHB AUC between trials. Postprandial NEFA concentrations were 14% lower in the high energy turnover trial compared to the low energy turnover trial. Postprandial glucose and insulin concentrations were 7% and 62% higher, respectively, in the high energy turnover trial compared to the low energy turnover trial. Calculation of the incremental AUC revealed no differences in TG responses between trials. The Incremental AUC for NEFA and 3-OHB was 104% and 129% lower, respectively, in the high energy turnover trial and for glucose and insulin 219% and 87% higher, respectively, in the high energy turnover trial compared to the low energy turnover trial.

Table 6.3 Summary postprandial plasma responses

	Low energy turnover	**High energy turnover**	***P***
Triglyceride (mmol.l^{-1})			
Time-averaged AUC	0.74 ± 0.05	0.79 ± 0.06	0.145
Incremental AUC	0.04 ± 0.02	0.08 ± 0.04	0.184
NEFA (mmol.l^{-1})			
Time-averaged AUC	0.37 ± 0.02	0.32 ± 0.02	0.009
Incremental AUC	-0.23 ± 0.04	-0.47 ± 0.04	0.003
3-hydroxybutyrate (µmol.l^{-1})			
Time-averaged AUC	110.28 ± 21.62	80.96 ± 10.70	0.143
Incremental AUC	-135.18 ± 56.02	-309.66 ± 59.73	0.029
Glucose (mmol.l^{-1})			
Time-averaged AUC	5.04 ± 0.15	5.38 ± 0.10	0.023
Incremental AUC	0.21 ± 0.15	0.67 ± 0.12	0.010
Insulin (mU.l^{-1})			
Time-averaged AUC	12.69 ± 1.45	20.49 ± 1.97	0.002
Incremental AUC	9.06 ± 1.34	16.97 ± 1.96	0.002

N=13, values are mean ± SEM. AUC: area under the six hour concentration *vs*. time curve, NEFA: non-esterified fatty acids.

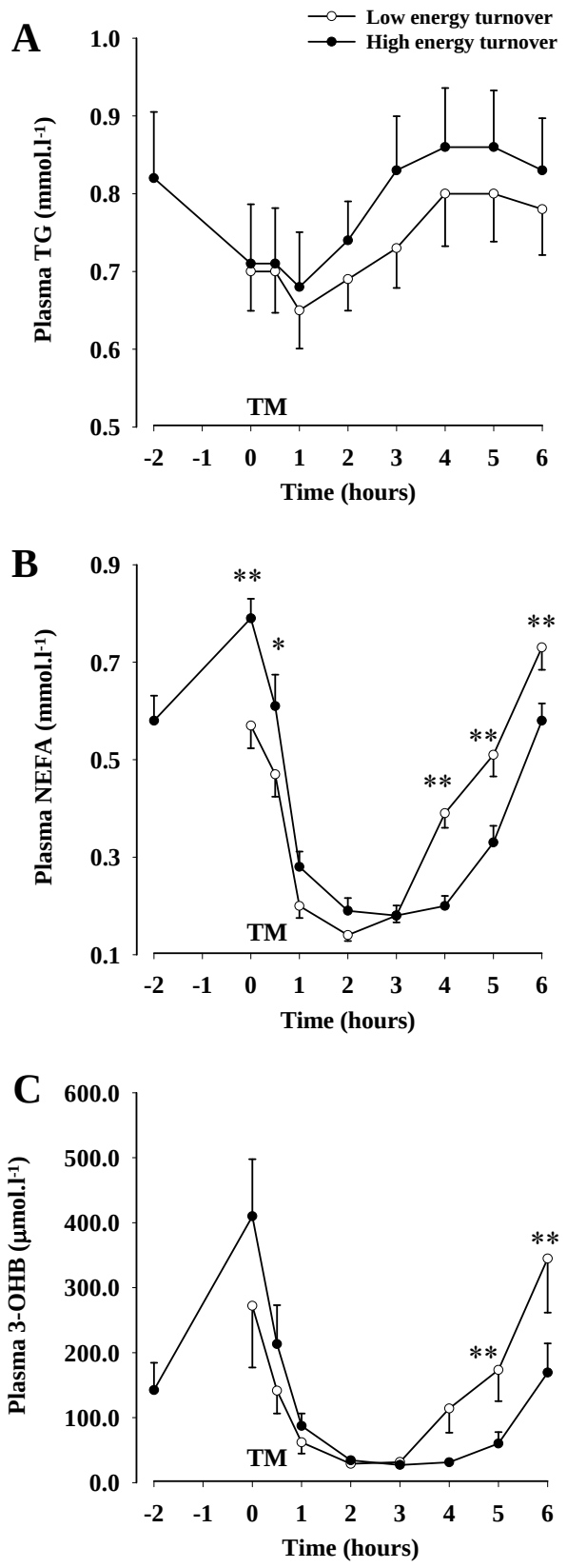


Figure 6.2 (A) Plasma triglyceride (TG), (B) non-esterified fatty acid (NEFA) and (C) 3-hydroxybutyrate (3-OHB) postprandial responses during the low energy turnover (o) and high energy turnover (●) trials. TM indicates the time at which the test meal was provided. ** $p < 0.01$, * $p < 0.05$.

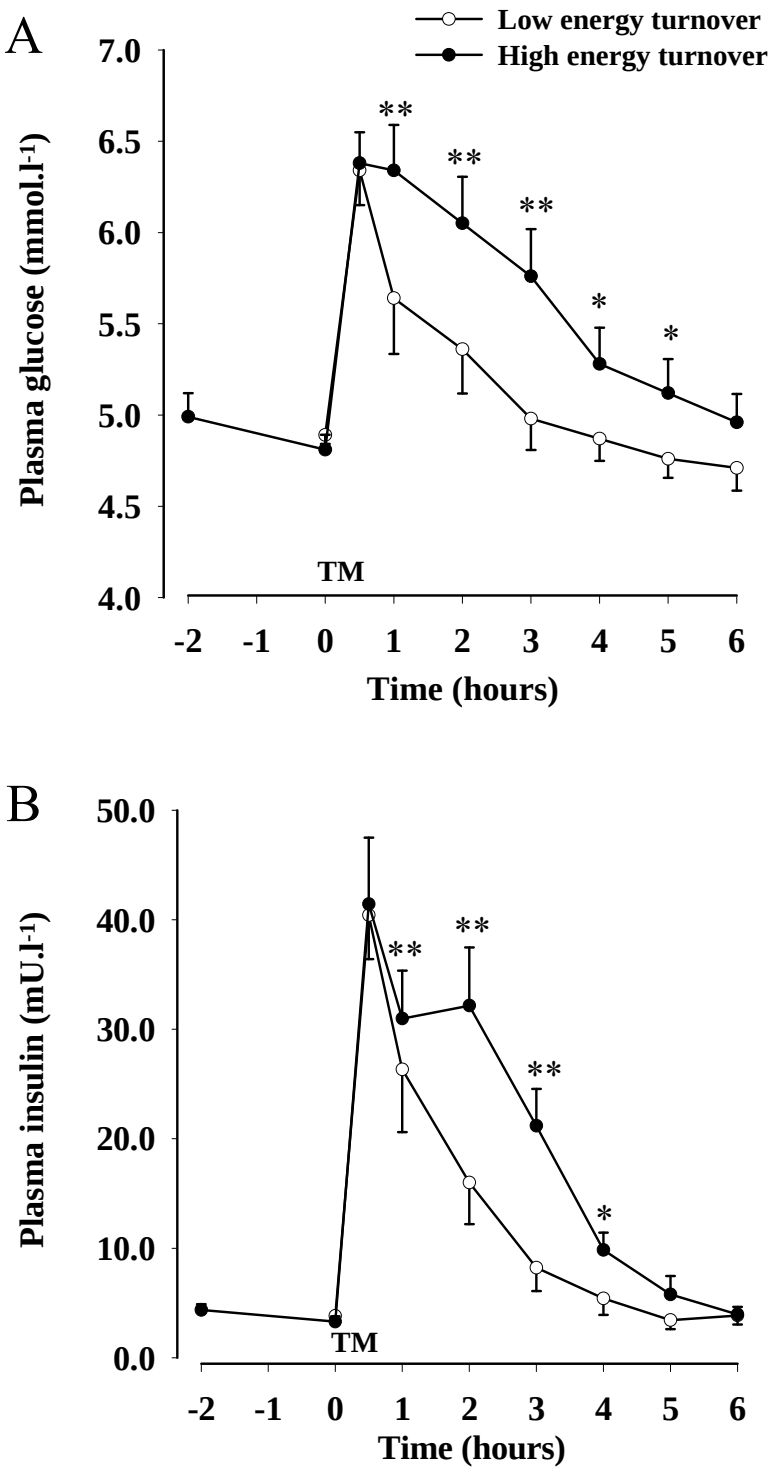


Figure 6.3 Plasma glucose (A) and insulin (B) postprandial responses during the low energy turnover (o) and high energy turnover (●) trials. TM indicates the time at which the test meal was provided. ** $p < 0.01$, * $p < 0.05$.

Figure 6.4 shows postprandial energy expenditure and substrate oxidation for both trials. The total energy expended during the six-hour postprandial observation period (i.e. 0 to 6 hours) was 1472 ± 37 kJ and 1550 ± 45 kJ in the low and high energy turnover trials, respectively ($p < 0.01$). The non-protein RQ was 0.75 ± 0.01 in the low energy turnover trial, 0.75 ± 0.01 in the high energy turnover trial and did not differ between trials ($p > 0.05$). There was no significant difference in total fat oxidation (low energy turnover trial: 18.7 ± 1.6 g, high energy turnover trial: 20.1 ± 1.5 g, $p > 0.05$) or carbohydrate oxidation (low energy turnover trial: 26.5 ± 3.5 g, high energy turnover trial: 28.9 ± 3.5 g, $p > 0.05$) between trials. Two-way analysis of variance however revealed trial x time differences in substrate oxidation (Figure 6.4); fat oxidation was significantly higher for three hours after the test meal in the high energy turnover trial compared to the low energy turnover trial, whereas carbohydrate oxidation was significantly higher in the high energy turnover trial during the later stages, at four and five hours, postprandially.

Neither the systolic nor diastolic blood pressure time-averaged AUCs were different between trials (systolic blood pressure; low energy turnover: 108 ± 4 mmHg, high energy turnover: 108 ± 4 mmHg, $p > 0.05$ and diastolic blood pressure; low energy turnover: 66 ± 2 mmHg, high energy turnover: 67 ± 2 mmHg, $p > 0.05$). No significant differences were observed in the time-averaged femoral PWV AUC (low energy turnover: 6.85 ± 0.19 $m.s^{-1}$, high energy turnover: 6.93 ± 0.19 $m.s^{-1}$, $p > 0.05$) or radial PWV AUC (low energy turnover: 8.85 ± 0.23 $m.s^{-1}$, high energy turnover: 9.14 ± 0.33 $m.s^{-1}$, $p > 0.05$) between the high and low energy turnover trials.

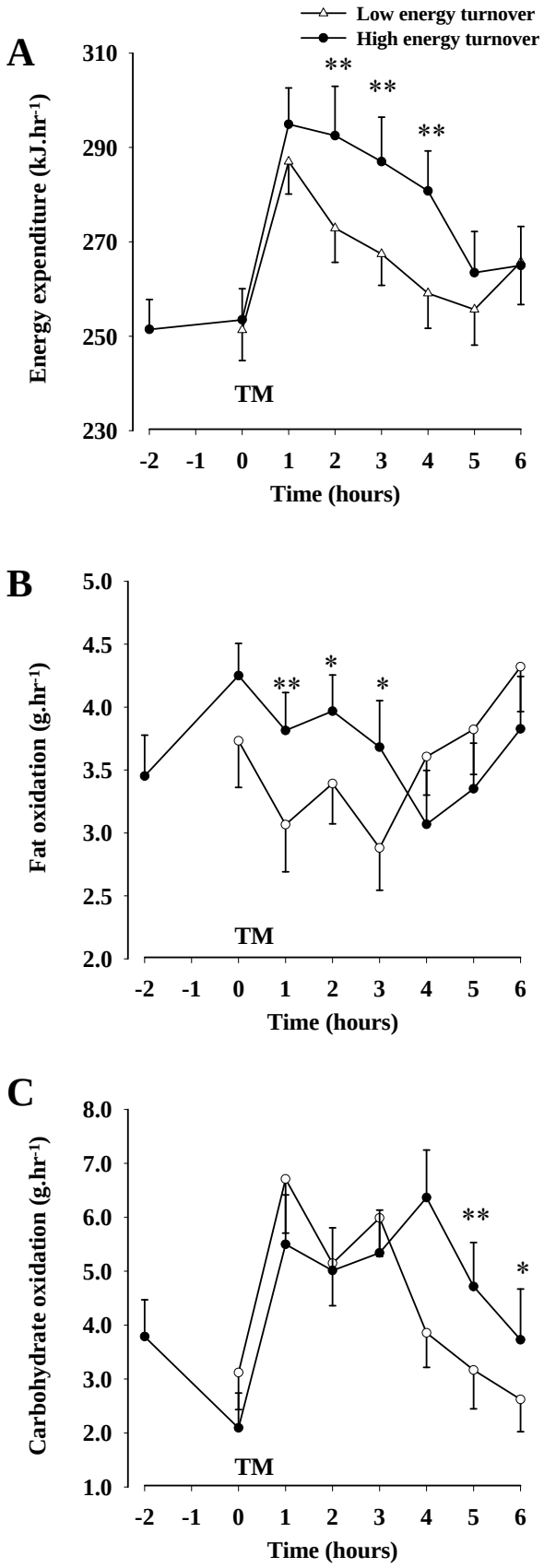


Figure 6.4 Postprandial energy expenditure (A), fat oxidation (B) and carbohydrate oxidation (C) during the low energy turnover (o) and high energy turnover (●) trials. TM Indicates the time at which the test meal was provided. ** $p < 0.01$, * $p < 0.05$.

6.3.4 Relationships between variables

There were no significant correlations between age, BMI or waist circumference and any of plasma TG, NEFA, 3-OHB, glucose or insulin in the fasting or postprandial state. Fat oxidation correlated with both NEFA ($r = 0.40$, $p < 0.05$) and 3-OHB ($r = 0.42$, $p < 0.05$) concentrations in the fasted state (**Figure 6.5**) but not during the postprandial period. Exercise-induced changes in postprandial TG responses were correlated with exercise-induced changes in both glucose ($r = 0.71$, $p < 0.01$) and insulin ($r = 0.56$, $p < 0.05$) responses. As expected, there was a strong correlation between exercise-induced changes in postprandial glucose and insulin ($r = 0.62$, $p < 0.05$) concentrations. Exercise-induced changes in whole body fat oxidation were associated with changes in both NEFA ($r = 0.56$, $p < 0.05$) and 3-OHB ($r = 0.73$, $p = 0.05$) concentrations (**Figure 6.6**).

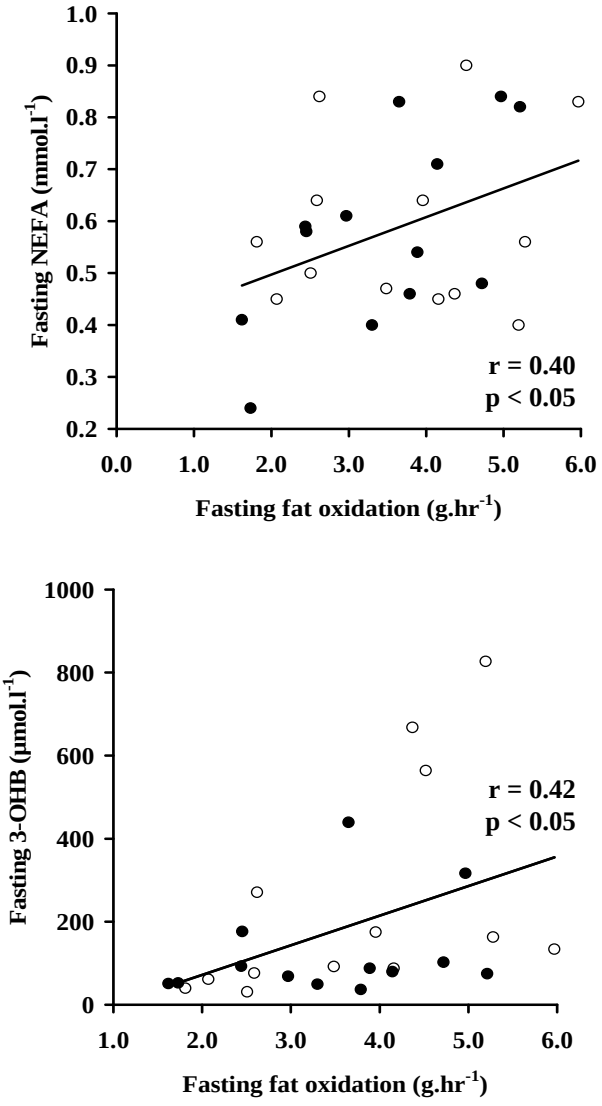


Figure 6.5 The relationship between fasting fat oxidation and fasting NEFA concentrations (top) and between fasting fat oxidation and fasting 3-OHB concentrations (bottom) in the high energy (●) and low energy (o) turnover trials.

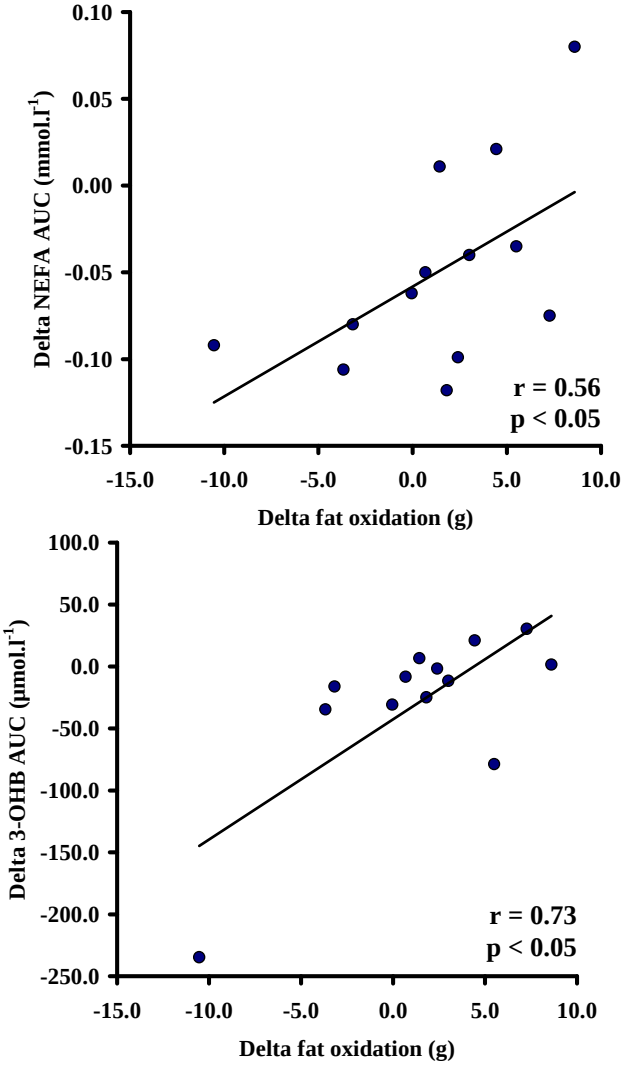


Figure 6.6 The relationship between the exercise-induced changes in postprandial fat oxidation and postprandial NEFA concentrations (top) and between the exercise-induced changes in postprandial fat oxidation and postprandial 3-OHB concentrations (bottom).

Neither fasting nor postprandial femoral or radial PWV correlated with age, BMI or waist circumference. Fasting femoral PWV correlated with diastolic blood pressure ($r = 0.44$, $p < 0.05$) but not systolic blood pressure. Fasting radial PWV did not correlate with either systolic or diastolic blood pressure. Post-exercise radial PWV strongly correlated with post-exercise diastolic blood pressure ($r = 0.83$, $p < 0.001$). Postprandially, femoral PWV was associated with systolic blood pressure ($r = 0.55$, $p < 0.01$) and, although less strongly, diastolic blood pressure ($r = 0.34$, $p < 0.05$) (**Figure 6.7**). Postprandial radial PWV correlated with diastolic blood pressure ($r = 0.56$, $p < 0.01$) and showed a strong tendency to also correlate with systolic blood pressure ($r = 0.38$, $p = 0.052$) (**Figure 6.8**). No

correlation was observed between femoral or radial PWV and TG in either the fasting or postprandial state.

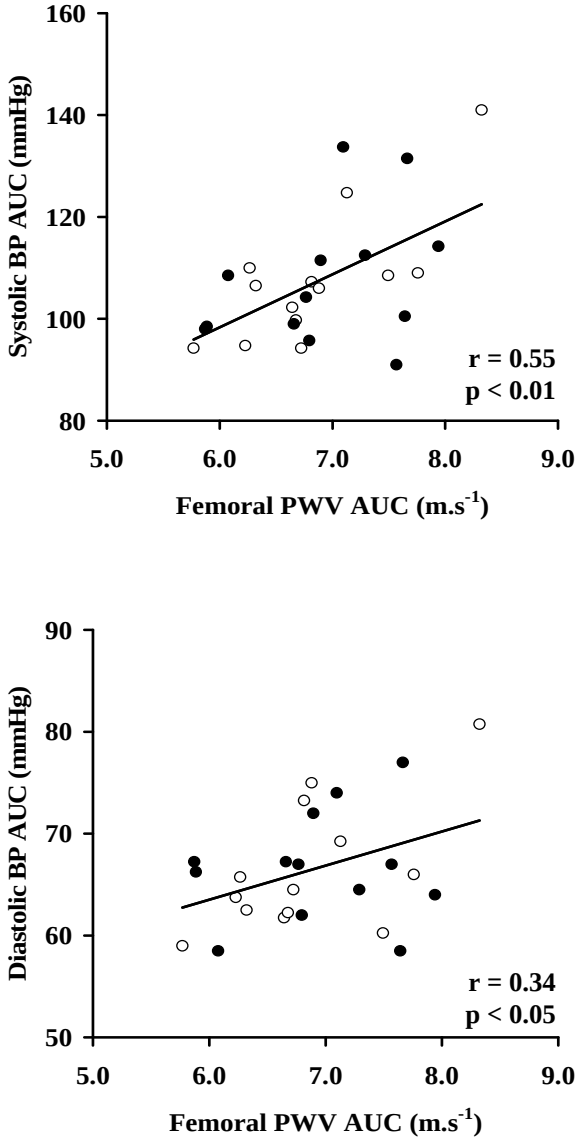


Figure 6.7 The relationship between postprandial femoral PWV and postprandial systolic blood pressure (top) and between postprandial femoral PWV and postprandial diastolic blood pressure (bottom) in the high energy (●) and low energy (o) turnover trials.

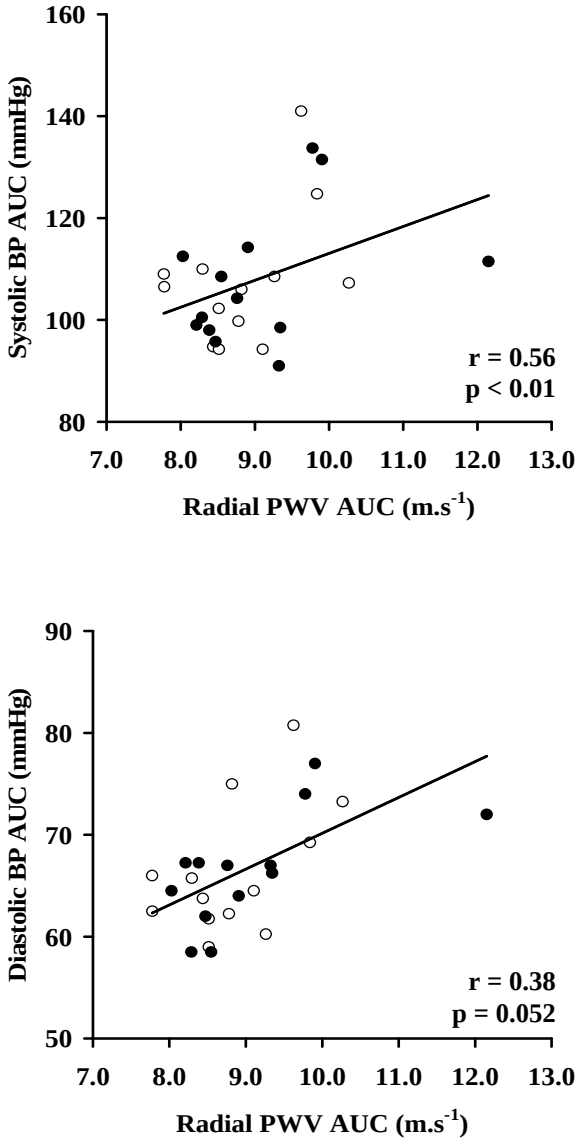


Figure 6.8 The relationship between postprandial radial PWV and postprandial systolic blood pressure (top) and between postprandial radial PWV and postprandial diastolic blood pressure (bottom) in the high energy (●) and low energy (O) turnover trials.

6.4 Discussion

The present study aimed to compare differences in postprandial metabolism and arterial stiffness between a state of energy balance with either high or low energy turnover. Results showed that in the high energy turnover trial the postprandial lipaemic, glycaemic and insulinaemic responses were all increased. Postprandial energy expenditure was elevated in the high energy turnover trial, with greater whole body fat oxidation during the initial three hours of the postprandial period. Whole body carbohydrate oxidation was significantly higher during the later stages of the high energy turnover trial. There was no

effect of trial on either systolic or diastolic blood pressure and similarly, neither femoral nor radial PWV were different between the two trials.

6.4.1 Postprandial metabolism

In Chapter 3 it was shown that although an exercise-induced energy deficit was important for maximising the benefits of exercise, it was not the sole determinant of all subsequent changes in postprandial metabolism. Postprandial insulinaemia was lower and whole body fat oxidation was higher after exercise with energy replacement, suggesting that energy balance when coupled with exercise-induced high energy turnover was metabolically different to energy balance at rest. Data from the present study further support this hypothesis, although with some interesting and unexpected findings.

The day following exercise, postprandial TG concentrations are attenuated, a response evident in trained and untrained individuals (Tsetsonis *et al.*, 1997), the overweight and obese (Gill *et al.*, 2004) and adolescents (Barrett *et al.*, 2007). The present study failed to show any attenuation of postprandial TG concentrations and in fact the postprandial TG response tended to be higher in the high energy turnover trial. Exercise-induced changes in TG do occur after a delay of some hours (Malkova & Gill, 2006) and measuring TG for six hours immediately post-exercise may explain why no effect was observed in the present study. The higher postprandial TG concentrations in the high energy turnover trial could conceivably reflect the greater amount of food that was provided in the test meal; consuming larger quantities of fat (Dubois *et al.*, 1998) and carbohydrate (Koutsari *et al.*, 2001) are known to augment postprandial lipaemia and in studies where the same meal is provided in either an exercise or control trial, there is no rise in TG following exercise (Clegg *et al.*, 2007). Fasting NEFA concentrations were increased immediately post-exercise and during the initial postprandial period in high energy turnover, a response that may reflect post-exercise elevations in fatty acid mobilisation from adipose tissue, that were not immediately balanced by an increase in the uptake and utilisation of fatty acids within muscle, subsequently increasing plasma NEFA concentrations (Mulla *et al.*, 2000). The rapid decline in postprandial NEFA concentrations up to two hours after the test meal may be a consequence of the elevated postprandial glucose and insulin concentrations observed during the high energy turnover trial, which are reported to suppress the mobilisation of NEFA from adipose tissue (Ferrannini *et al.*, 1983).

An interesting finding of the present study was the augmented postprandial glucose and insulin responses in high energy turnover, perhaps suggesting an increase in insulin

resistance. Postprandial glucose concentrations often remain unchanged the day following exercise, as shown in Chapter 3 and earlier studies (Gill *et al.*, 2001b;Tsetsonis & Hardman, 1996), and immediately post-exercise (Katsanos & Moffatt, 2004;Malkova *et al.*, 2000). Postprandial insulin concentrations are typically reported to be either stable or lower the day after exercise (Gill *et al.*, 2002b;Malkova *et al.*, 2000;Tsetsonis *et al.*, 1997) and during the immediate post-exercise period (Englert *et al.*, 2006;Katsanos & Moffatt, 2004). In Chapter 3, a significantly lower insulin response was observed on the day following exercise with energy replacement, suggesting that exercise might mediate insulin metabolism via different mechanisms to energy deficit. It is unclear therefore, why, in the present study, postprandial glucose and insulin responses were elevated. Immediate short-term increases in postprandial glucose and insulin concentrations in the high energy turnover trial were expected because of the extra food provided in the test meal, however, both glucose and insulin typically return to near baseline values within approximately three hours after a meal (Frayn *et al.*, 1993) and as such are unlikely to explain the elevated postprandial glucose and insulin response measured at five hours in the high energy turnover trial. To the author's knowledge, only one previous study in humans (Broom *et al.*, 2007) has reported an elevated glucose concentration following exercise and no explanation was provided for such a finding. In the present study it is likely that the elevated glucose concentrations are a consequence of the glucose-fatty acid cycle (Randle *et al.*, 1963) causing glucose to build up in the blood as a consequence of the preferential uptake and oxidation of fatty acids. Provision of the extra food in the present study to replace the energy expended is likely to have exacerbated the high glucose concentrations, which may explain why few studies have previously reported such an effect. With regard to the elevated insulin response, again to the author's knowledge there has been no previous report of such a response and therefore it is perhaps feasible to suggest that the rise in insulin was a secondary response to the elevated glucose concentrations.

Postprandial energy expenditure was 5% higher in the high energy turnover trial which is consistent with the earlier findings of Votruba et al. (Votruba *et al.*, 2005), although these authors preserved the exercise-induced energy deficit. Indeed, excess post-exercise oxygen consumption (EPOC) (i.e. increased post-exercise energy expenditure) can persist for up to 24 hours post-exercise (Laforgia *et al.*, 2006). A review by Borsheim and Bahr (Borsheim & Bahr, 2003) suggested that resting metabolic rate might be elevated from between 3% to 10% following a single session of exercise, which is consistent with the findings of this study. It should however be remembered that an extra 10% of the energy expended during exercise was provided to account for such metabolic consequences of the

EPOC period and therefore minimising its effect. It is also possible that the thermic effect of the extra food provided in the high energy turnover trial (Reed & Hill, 1996) may have contributed to the elevated rate of energy expenditure, and an earlier study has in fact shown that in overweight men and women with a mean age of 45 years, dietary-induced thermogenesis increased by 5.2% following a mixed meal providing approximately 2.3 MJ of energy (Westerterp-Plantenga *et al.*, 1997b).

Immediately post-exercise, fasting fat oxidation was higher and carbohydrate oxidation lower compared to pre-exercise values. These findings are consistent with other groups also reporting higher fat oxidation during immediate post-exercise recovery (Votruba *et al.*, 2002;Votruba *et al.*, 2005). The higher post-exercise NEFA concentrations in the current study may have facilitated an increase in fat oxidation (Frayn, 2003), with a significant correlation of 0.40 between the two variables. Furthermore a rise in 3-OHB concentrations, indicative of greater hepatic fatty acid oxidation (Williamson & Whitelaw, 1978), was also reported which may have contributed to the higher whole body fat oxidation (corrletaion of 0.42 between fat oxidation and 3-OHB concentrations). Earlier studies have shown postprandial fat oxidation to also be increased during the immediate post-exercise recovery period (Votruba *et al.*, 2002;Votruba *et al.*, 2005) and the day following exercise (Hansen *et al.*, 2005). Chapter 3 revealed higher rates of fat oxidation the day after exercise with and without energy replacement. Considering the evidence therefore, the finding of the current study that neither fat nor carbohydrate oxidation differed between trials was somewhat surprising, although the lower postprandial NEFA response, and therefore lower substrate availability, observed in high energy turnover may have inhibited the expected increase in fat oxidation during the later stages of the postprandial period. In the absence of any trial effect, trial by time interactions did show postprandial fat oxidation to be significantly higher for three hours after the high energy turnover test meal, during which time postprandial NEFA concentrations were also some what elevated. Although the present study can not propose a direct mechanism for such a short term effect, exercise-induced changes in postprandial fat oxidation were correlated with changes in 3-OHB concentrations, which appear independent of any change in NEFA availability with no relationship observed between postprandial NEFA and 3-OHB concentrations. Changes in 3-OHB explained 53% of the variation in whole body fat oxidation, thus although mean postprandial fat oxidation did not change, a short term increase in fat oxidation in the high energy turnover trial may have been mediated, at least in part, by an increase in hepatic fatty acid oxidation. Fat oxidation within other tissues such as skeletal muscle (Kiens & Richter, 1998) is however also likely to be important.

6.4.2 Pulse wave velocity

Pulse wave velocity provides an index of endothelial function and arterial stiffness (Asmar *et al.*, 1997;Boutouyrie *et al.*, 2002). In Chapter 4 it was shown that radial PWV was lower the day following exercise both with and without energy replacement, suggesting that exercise *per se* rather than the associated energy deficit mediated the lower PWV. To further understand the effects of exercise with energy replacement, the current study measured femoral and radial PWV responses during the high and low energy turnover trials. No immediate effect of exercise was observed in post-exercise femoral PWV, but radial PWV was significantly lower compared to pre-exercise values, data which are consistent with those from Naka *et al*. (Naka *et al.*, 2003) and Kingwell *et al*. (Kingwell *et al.*, 1997b). The strong correlation observed between post-exercise radial PWV and post-exercise diastolic blood pressure suggests that blood pressure may have been an important mediator of such a response.

During the postprandial period, there were no differences in femoral or radial PWV between trials, suggesting that energy balance with high energy turnover has no greater effect on PWV than low energy turnover, findings which are quite different to those presented in Chapter 4. Different experimental timings used in this study and in Chapter 4, may explain the variation in the results; a change in PWV may occur only after a delay of some hours. Further support for a delayed PWV response is provided by the relationship between postprandial PWV and TG concentrations as shown in Chapter 4 and the literature (Daskalova *et al.*, 2005). Exercise-induced changes in TG concentrations occur after a delay of a number of hours (Malkova & Gill, 2006), and the absence of any attenuation in TG in the current study may have inhibited any subsequent change in femoral or radial PWV. However, it is also possible that differences in the subject groups used in this study; young, overweight, pre-menopausal women and the study in Chapter 4; middle-aged, obese men, may also explain the conflicting findings. There is a need for continued research to further investigate how variables such as age, gender and body weight may determine the effects of exercise in a state of energy balance on PWV.

It is less clear why the present study failed to show any change in PWV during the short-term post-exercise recovery period whereas earlier studies report lower central and peripheral PWV after acute exercise (Heffernan *et al.*, 2007a;Kingwell *et al.*, 1997b;Naka *et al.*, 2003). That the current study replaced the energy expended during exercise is an obvious difference, but data from Chapter 4 suggested that the energy deficit did not mediate the exercise-induced lowering of PWV, at least the day following exercise. The

absence of exercise with an energy deficit in the present study does, however, make it difficult to extend the findings of Chapter 4 to this study, and it is possible that an exercise-induced energy deficit may be required to induce short-term changes in femoral and radial PWV. The association between blood pressure and PWV (Asmar *et al.*, 1997) is also important. The current data reported postprandial femoral PWV to correlate with systolic and diastolic blood pressure and although radial PWV was significantly correlated with only diastolic blood pressure, there was a strong tendency for a correlation with systolic pressure as well. Acute changes in blood pressure have been shown to attenuate PWV (Stewart *et al.*, 2006) and thus the absence of any change in either postprandial systolic or diastolic blood pressure between trials may explain the absence of any change in PWV. Finally, it should be made aware that in the current study, both femoral and radial PWV were measured using an automated Complior device, whereas Doppler flow probes (Heffernan *et al.*, 2007a;Kingwell *et al.*, 1997b) and oscillometry (Naka *et al.*, 2003) have been used previously. These methodological differences should be considered when interpreting data from the current study with that from others (Hamilton *et al.*, 2007).

In summary, data from the present study suggest that a state of energy balance with high energy turnover is metabolically different to a state of energy balance with low energy turnover. Postprandial lipaemia, glycaemia and insulinaemia were all elevated in a state of high energy turnover, whereas no effect was observed in either femoral or radial PWV. High energy turnover increased the rate of postprandial energy expenditure and whole body fat oxidation although such an effect was attenuated during the later postprandial stages, during which time an elevation in carbohydrate oxidation occurs. These shifts in energy expenditure and substrate metabolism may have important implications for future weight regulation. Further research is warranted to investigate how energy balance and energy turnover affect postprandial metabolism in other populations including overweight and obese men, patients with type 2 diabetes, adolescents and younger children.

CHAPTER 7
ENERGY BALANCE WITH HIGH AND LOW ENERGY TURNOVER: IMPLICATIONS FOR APPETITE REGULATION AND FEEDING BEHAVIOUR

7.1 Introduction

Individuals who regularly exercise appear to exert a tighter coupling between energy expenditure and energy intake compared to their sedentary peers and as such, are better able to maintain stable body weights and resist weight gain (King *et al.*, 1997b;Wareham *et al.*, 2005). This suggests that over a relatively prolonged period of time, those participating in regular exercise are better able to maintain energy balance, evidence which again may suggest that states of energy balance with high or low energy turnover differ.

An exercise-induced high energy turnover with energy deficit might regulate body weight via its effect on appetite control and feeding behaviour, a hypothesis which has previously been addressed. Following moderate to high intensity exercise, hunger is suppressed (Burns *et al.*, 2007;King *et al.*, 1994;Westerterp-Plantenga *et al.*, 1997a) and satiety increased (Tsofliou *et al.*, 2003), although some have failed to report any effect of exercise on appetite (Hubert *et al.*, 1998;Imbeault *et al.*, 1997). Measuring food intake has fairly consistently shown that energy intake remains unchanged following acute exercise (George & Morganstein, 2003;Hubert *et al.*, 1998;Imbeault *et al.*, 1997;King *et al.*, 1997a) although relative energy intake, energy intake in relation to the energy expended during exercise, is lower (Imbeault *et al.*, 1997;Martins *et al.*, 2007a;Pomerleau *et al.*, 2004). Over more prolonged periods of time, however, evidence suggests that some degree of compensation for the exercise-induced energy expenditure may occur (Stubbs *et al.*, 2002b). Exercise may also alter macronutrient intake but again the literature is inconsistent with some showing fat (Pomerleau *et al.*, 2004) and others carbohydrate (Stubbs *et al.*, 2004;Westerterp-Plantenga *et al.*, 1997a) to be preferentially consumed and others observing no difference in macronutrient consumption (Hubert *et al.*, 1998;King *et al.*, 1997a). It was believed that exercise-induced changes in ghrelin, an orexigenic hormone stimulating hunger and food intake (Druce *et al.*, 2005;Wren *et al.*, 2001), may modulate appetite. Previous literature has, however, consistently failed to show any response of total ghrelin to acute exercise (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a) suggesting that changes in ghrelin do not contribute to changes in appetite control and feeding behaviour. Recent data does though suggest that it is not total ghrelin *per se* but rather the ratio of acylated ghrelin to de-acylated ghrelin that may be altered following exercise (Broom *et al.*, 2007;Mackelvie *et*

al., 2007), a change that would not have been observed when only total ghrelin was measured. Therefore it is also possible that changes in acylated ghrelin, not total ghrelin, are related to changes in appetite during the post-exercise period (Broom *et al.*, 2007).

As suggested above, appetite regulation may differ in a state of energy balance compared to a state of energy deficit. Thus, the fact that previous studies investigating the association between exercise and markers of appetite have typically been conducted in an energy deficit state, with no attempt to maintain energy balance, is an important consideration, making it difficult to distinguish between the effects of exercise *per se* or the associated energy deficit on appetite regulation. This limitation was addressed in Chapter 5, where the effects of exercise, with and without energy replacement, on appetite and appetite hormones were measured the day following exercise. Neither exercise with nor without energy replacement altered hunger, satiety, fullness, PFC or desire to eat in obese middle-aged men, although no direct measure of energy or macronutrient intake was used. Both exercise interventions also failed to attenuate total ghrelin concentrations, but the effect on acylated ghrelin was not measured. In Chapter 5, the 16-hour delay between exercise and the determination of appetite and appetite hormones will likely have overlooked any immediate effect of exercise on appetite. Therefore, and consistent with study protocols used in earlier studies, measuring the immediate short term appetite responses to exercise during a state of energy balance will help understanding of the mechanisms by which exercise regulates appetite.

The present study was designed to compare how a state of energy balance with high energy turnover i.e. energy balance with exercise, differs from a state of energy balance with low energy turnover i.e. energy balance with rest. To achieve this, the immediate effects of exercise or rest, with a state of energy balance maintained, on appetite, 'hunger' hormones and feeding behaviour were investigated.

7.2 Methods

Thirteen pre-menopausal women were recruited into this study. The average age, BMI, waist circumference and maximal oxygen consumption of the women were 32 ± 8 years, 27.5 ± 1.1 $kg.m^{-2}$, 87.1 ± 6.2 cm and 34.7 ± 5.8 $ml.kg^{-1}.min^{-1}$, respectively (mean ± SD). All of the women were apparently healthy, non-smoking and with no known cardiovascular disease or diabetes. None was taking any medication thought to interfere with lipid or energy substrate metabolism. Because one aim of this study was to measure energy and macronutrient intake during a buffet meal, the subject information provided

upon entry to the study was manipulated and subjects were informed that the aim was to investigate the effects of exercise on markers of food palatability. The author felt this was necessary to avoid changes in normal eating behaviour that can occur as a consequence of an individuals own perception of how much food is 'socially' acceptable to eat when being observed by others (Bock & Kanerek, 1995).

7.2.1 Preliminary sessions

Prior to experimental trials, individual subjects were asked to complete a questionnaire providing details of i) foods they did not like, ii) specific dietary requirements e.g. vegetarian and iii) all food allergies. This information was used to ensure foods provided in the buffet meal were suitable, and safe, for subjects to consume.

Each subject attended the metabolic suite having fasted overnight for at least 12 hours. After 20 minutes of rest lying on the couch, a 25-minute expired air sample was collected and resting metabolic rate was calculated using indirect calorimetry (Chapters 2.4.1 and 2.5). Daily energy requirements were calculated on an individual basis using a PAL of 1.2 (Chapter 2.4.1.1). Full anthropometric measurements were completed for each subject using the methods previously described in Chapter 2.3.

Each subject completed a four-stage sub-maximal fitness test to estimate their maximal oxygen consumption ($\dot{V}O_2$ max) and calculate the speed and gradient that was required to elicit an intensity of 50% $\dot{V}O_2$ max, the intensity that was used for the exercise in the high energy turnover trial (Chapter 2.7.1).

7.2.2 Experimental design

Each subject completed two separate one day trials; energy balance with low energy turnover (low energy turnover) and energy balance with high energy turnover (high energy turnover). Trials were performed in a randomised design and completed approximately four weeks apart to control for the menstrual cycle. An overview of the study protocol is shown in **Figure 7.1**. For three days prior to each experimental trial, subjects were instructed to adhere to strict pre-trial controls as described in Chapter 2.9.

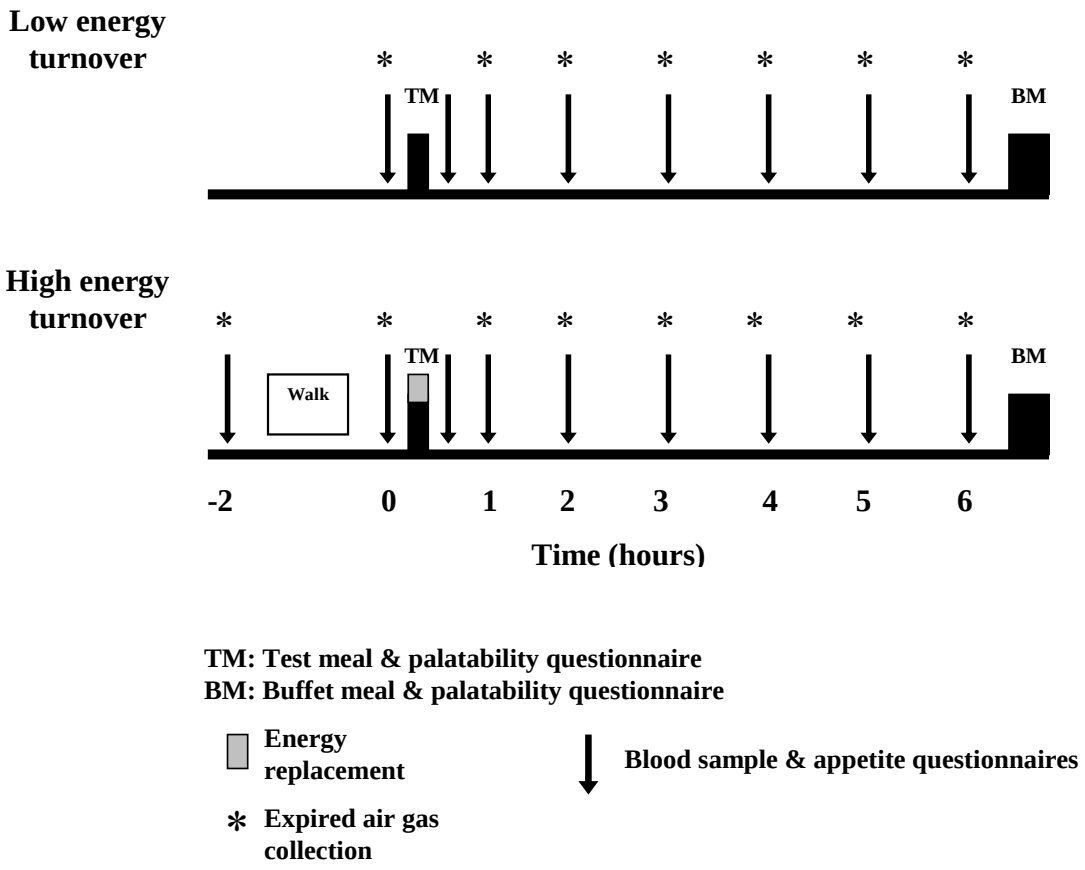


Figure 7.1 An overview of the experimental study design

7.2.3 Low energy turnover trial

Subjects attended the metabolic suite at 10:00 am following an overnight fast starting at 8:00 pm the previous evening. Fasting expired air measurements (Chapter 2.4.1) and blood samples (Chapter 2.8.2.1) were collected and a fasting VAS appetite questionnaire was completed as described in Chapter 2.8.3. On completion of all fasting measurements, the subject was provided with the test meal (Chapter 2.8.1). During the postprandial metabolic assessment period, blood samples and appetite questionnaires were collected at 0.5, 1, 2, 3, 4, 5 and 6 hours after the test meal. Expired air measurements were taken during the 15 minutes prior to the 1, 2, 3, 4, 5 and 6 hour blood samples (Figure 7.1). Thirty minutes after the final blood sample was taken (6.5 hours after the test meal), subjects were provided with a buffet meal as described below. During both the test meal and the buffet meal, subjects completed VAS food palatability questionnaires as described in Chapter 2.8.3.

7.2.4 High energy turnover trial

Subjects attended the metabolic suite at 8:00 am following an overnight fast of 12 hours that started at 8:00 pm the night before. A fasting expired air and blood sample was collected and a VAS appetite questionnaire completed. On completion of fasting measurements, subjects performed a 60 minute treadmill walk, for details of which the reader is referred to Chapter 6.2.4. On completion of the walk, subjects were allowed to shower and change, and fasting post-exercise expired air, blood samples and VAS appetite questionnaires were repeated (Figure 7.1). After the post-exercise measurements, the same test meal as that used in the low energy turnover trial was provided, but this time the energy content was increased to replace 110% of the net energy expended during the trial walk (Chapter 2.8.1). Postprandial blood samples, appetite questionnaires and expired air measurements were collected in exactly the same way as described above for the low energy turnover trial and as shown in Figure 7.1. Thirty minutes after the final blood sample was collected (6.5 hours after the test meal), subjects were provided with a buffet meal as described below. Again, during both the test meal and the buffet meal, subjects were asked to complete VAS food palatability questionnaires as described in Chapter 2.8.3.

7.2.5 Ad libitum buffet meal and measurement of energy and macronutrient intakes

The buffet meal was exactly the same in both trials. A variety of foods were offered, an overview of which is given in **Table 7.1**. The energy and nutrient content of each of the foods (per 100 gram) was taken, where possible, from the food manufacturers own dietary labels. If this information was not available, for example for cucumber and tomatoes, dietary information was provided by a computerised dietary analysis programme (CompEat Version 5, Nutrition Systems, Grantham, UK). Overall, 10% of energy in the buffet meal was provided by protein, 44% fat and 46% carbohydrate. All foods were individually weighed prior to each meal to provide the exact amount that was required. Foods were presented in the same dishes and using the same table layout in both trials and all foods were presented to the subject in quantities in excess of expected consumption. Subjects were taken to a separate room for the duration of the meal, where they remained alone to avoid the influence of being in a group environment on eating behaviour (Herman & Polivy, 2005). No music, television or reading material was permitted and no fluids were consumed. All subjects were instructed to eat until they had reached their normal level of satiation. A maximum time of 30 minutes was allowed for the meal, however subjects were free to return to the metabolic suite within this time if they had consumed

enough. Subjects were instructed to leave any food they had not eaten either on their plates or in the dishes in which they were originally provided.

Table 7.1 Dietary composition of the buffet meal.

	Mass (g)	**Energy (kJ)**	**Protein (g)**	**Fat (g)**	**Carbohydrate (g)**
Tomato & mozzarella pasta bake	780	3916	40.6	29.6	127.1
Lettuce salad	65	38	0.5	0.3	1.1
Cherry tomatoes	125	89	0.9	0.4	3.9
Cucumber	125	52	0.9	0.1	1.9
French baguette	175	1977	16.8	4.7	97.0
Butter	50	1550	0.5	40.8	0.5
Italian grated cheese	35	710	15.1	11.9	0.0
Salad dressing	200	905	0.2	18.6	10.1
Peach slices*	400	770	1.6	0.0	44.0
Fruit cocktail*	400	820	2.4	0.0	46.4
Chocolate cake	115	1944	5.2	21.2	63.5
Double cream	150	1856	3.1	45.8	5.0
Total		**14,627**	**87.8**	**173.4**	**400.5**

*tinned in fruit juice

To ensure subjects remained unaware that their food intake was being monitored no food was re-weighed until the subject had left the metabolic suite to return home. All of the foods, including any that remained on the subjects' plates, were re-weighed and the amount recorded. The weights of separate foods consumed were calculated as follows:

$$\text{Food intake (g)} = \text{Food provided (g)} - \text{Food consumed (g)} \quad \textbf{(Equation 7.1)}$$

The energy and nutrient composition of the foods were determined using information from the food manufacturers' labels or a dietary analysis programme (CompEat Version 5, Nutrition Systems, Grantham, UK).

7.2.6 Blood Analysis

Baseline total ghrelin and acylated ghrelin concentrations were determined in the fasting and post-exercise states in the low energy turnover and high energy turnover trials, respectively. Postprandial total ghrelin and acylated ghrelin concentrations were made throughout the postprandial observation period. At the same time points, plasma glucose and insulin concentrations were also measured. A detailed description of all these analysis is given in Chapter 2.10.

7.2.7 Data analysis

Energy expenditure and energy substrate utilisation, corrected for protein oxidation, were calculated using indirect calorimetry (Chapter 2.5). The total AUC, calculated using the trapezium rule, divided by the duration of the observation period (six hours) i.e. the time-averaged AUC were used as summary measures of the postprandial responses. Cumulative energy and energy substrate balances were calculated by summing the AUC for individual time periods throughout each experimental trial (-2 to -1.75 hours, -1.75 to -1.5 hours, -1.5 to -1.25 hours, -1.25 to -1 hours, -1 to 0 hours, 0 to 0.16 hours, 0.16 to 1 hours, 1 to 2 hours, 2 to 3 hours, 3 to 4 hours, 4 to 5 hours, 5 to 6 hours, 6 to 6.5 hours and 6.5 to 6.75 hours).

Data were analysed using Statistica (version 6.0, Statsoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennyslyvania). Prior to analysis all data were tested for normality and if necessary, logarithmically transformed. Subsequently, acylated ghrelin concentrations were transformed prior to statistical analysis. Differences between trials for fasting and post-exercise values and summary responses were analysed using paired samples t-tests. Differences over time for the two trials were calculated using two-way ANOVA with repeated measures for trial and time. Post hoc Fisher least significant difference tests were used to identify where differences lay when main trial and or interaction effects were observed. Relationships between variables were assessed using Pearson product-moment correlations. Statistical significance was accepted as $p < 0.05$ and data are presented as mean ± SEM unless otherwise stated.

Chapter 7.3 Results

7.3.1 Responses during the treadmill walk

The responses during the treadmill walk have been previously reported in Chapter 6, however briefly, subjects walked for one hour at a speed of 5.5 ± 0.1 $km.h^{-1}$ up a gradient of 2.4 ± 0.6%, rating the exercise as 'light' (level 11.1 ± 0.3) using the Borg scale (Borg,

1973). Mean $\dot{V}O_2$ and heart rate during the walk were 16.6 ± 0.7 ml.kg^{-1}.min^{-1} and 123 ± 2 beats.minute^{-1}, respectively. The net energy expenditure, fat oxidation and carbohydrate oxidation during the walk were 1.18 ± 0.07 MJ, 22.3 ± 1.4 g and 18.1 ± 3.3 g, respectively.

7.3.2 Ad libitum energy and macronutrient intake

Energy, protein, total fat and total carbohydrate intake at the buffet meal for both the low and high energy turnover trials are given in **Table 7.2**. Energy intake and fat intake did not differ between trials. Although it did not achieve statistical significance, carbohydrate intake tended to be 8% lower in the high energy turnover trial. There were also no differences between trials in the percentage of energy consumed from protein (low energy turnover: 13.3 ± 0.4%, high energy turnover: 13.3 ± 0.4%, $p > 0.05$), fat (low energy turnover: 30.4 ± 1.3%, high energy turnover: 31.2 ± 1.3%, $p > 0.05$) or carbohydrate (low energy turnover: 57.3 ± 1.1%, high energy turnover: 56.5 ± 1.1%, $p > 0.05$).

Table 7.2 Energy and macronutrient intake during the ad libitum buffet meal.

	Low energy turnover	**High energy turnover**	***P***
Energy (MJ)	3.8 ± 0.2	3.5 ± 0.2	0.105
Protein (g)	29.2 ± 1.3	27.4 ± 1.9	0.203
Total fat (g)	31.0 ± 1.9	29.1 ± 1.3	0.208
Total carbohydrate (g)	128.8 ± 5.9	118.7 ± 6.7	0.084

N = 13, values are mean ± SEM.

Cumulative energy, fat and carbohydrate balance during each trial are shown in **Figure 7.2**. Cumulative energy balance did not differ between trials during the postprandial observation period, prior to the buffet. After the buffet meal, however, cumulative energy balance was significantly lower in the high energy turnover trial compared to the low energy turnover trial (3.41 ± 0.16 MJ vs. 3.13 ± 0.15 MJ for the low and high energy turnover trials, respectively, $p < 0.001$). Cumulative fat balance was lower during the postprandial observation period in the high energy turnover trial and remained lower after consumption of the buffet meal (low energy turnover: 17.7 ± 3.1 g, high energy turnover: 6.1 ± 2.7g, $p < 0.001$). Carbohydrate balance was higher during the high energy turnover trial postprandial observation period but after consuming the buffet meal, there was no longer any difference in carbohydrate balance between the trials (low energy turnover: 151.8 ± 7.2, high energy turnover: 152.5 ± 4.4g, $p > 0.05$).

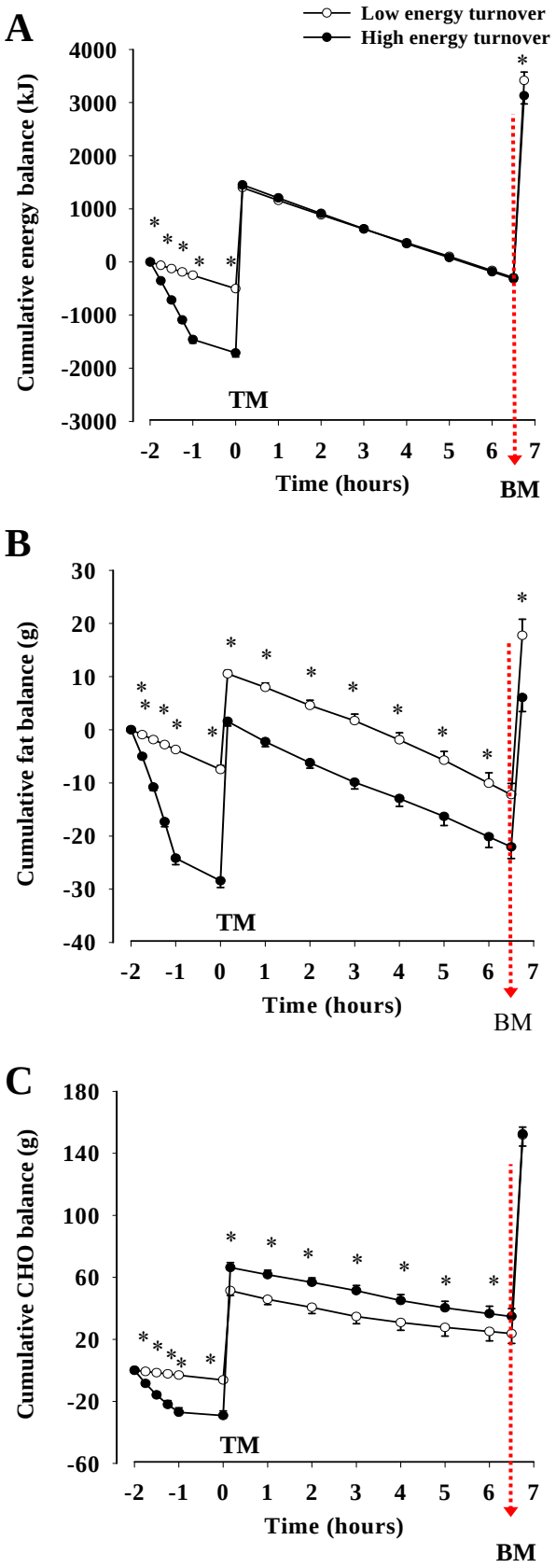


Figure 7.2 (A) Cumulative energy balance, (B) cumulative fat balance and (C) cumulative carbohydrate (CHO) balance during the low energy turnover (o) and high energy turnover (●) trials. TM and BM (⋮) indicate the time at which the test meal and buffet meal, respectively, were provided. * Significant difference between trials, $p < 0.001$.

7.3.3 Plasma metabolic variables and subjective ratings of appetite in the fasted state

Readers are referred back to Chapter 6.3 for the fasting glucose and insulin responses in the high and low energy turnover trials. Due to technical problems, total ghrelin and acylated ghrelin were unable to be determined in two subjects, therefore the data reported are for 11 subjects rather than 13. Low energy turnover fasting and high energy turnover post-exercise total ghrelin concentrations were not different (low energy turnover: 0.95 ± 0.16 $ng.ml^{-1}$, high energy turnover: 1.17 ± 0.48 $ng.ml^{-1}$, $p > 0.05$). High energy turnover post-exercise acylated ghrelin was however 23% lower compared to low energy turnover fasting concentrations (low energy turnover: 99.6 ± 16.6 $pg.ml^{-1}$, high energy turnover: 76.8 ± 12.4 $pg.ml^{-1}$, $p < 0.05$).

Fasting and post-exercise appetite ratings are shown in **Table 7.3**. Fasting ratings of satiety and fullness were increased by 64% ($p < 0.05$) and 70% ($p < 0.05$), respectively, in the high energy turnover trial compared to the low energy turnover trial. No significant differences were observed in fasting ratings of hunger, PFC or desire to eat ($p > 0.05$ for all). Comparison of low energy turnover fasting and high energy turnover post-exercise ratings revealed no significant differences in hunger, satiety, fullness, PFC or desire ($p > 0.05$ for all). When comparing fasting and post-exercise appetite ratings within the high energy turnover trial, hunger was 32% higher ($p < 0.05$) and satiety 31% lower ($p < 0.05$) post-exercise compared to fasting. Ratings of fullness, PFC and desire were not different post-exercise ($p > 0.05$ for all).

Table 7.3 Subjective ratings of appetite in the fasting state

	Low energy turnover: fasting	**High energy turnover: fasting**	**High energy turnover: post-exercise**
Hunger (mm)	68.0 ± 4.2	53.4 ± 5.8	70.5 ± 3.7[b]
Satiety (mm)	20.5 ± 2.3	33.7 ± 4.7[a]	23.4 ± 4.4[b]
Fullness (mm)	17.2 ± 3.3	29.2 ± 5.3[a]	18.4 ± 3.9
PFC (mm)	68.9 ± 3.9	65.3 ± 4.4	73.0 ± 4.4
Desire (mm)	72.7 ± 4.0	57.1 ± 5.6	67.5 ± 5.0

N = 13, values are mean ± SEM. [a]different to low energy turnover: fasting ($p < 0.05$), [b]different to high energy turnover fasting ($p < 0.05$). PFC: prospective food consumption

7.3.4 Postprandial plasma metabolic variables and subjective ratings of appetite

Again, the reader is referred back to Chapter 6.3 for the postprandial glucose and insulin responses in the high and low energy turnover trials. **Figure 7.3** and **Figure 7.4** show the postprandial responses over the six hour metabolic assessment period for total ghrelin and acylated ghrelin, respectively. Data are presented n = 11. There were no significant differences in the total ghrelin response between trials (low energy turnover: 0.82 ± 0.21 $ng.ml^{-1}$, high energy turnover: 0.98 ± 0.26 $ng.ml^{-1}$, $p > 0.05$). The postprandial acylated ghrelin response was however, 19% lower in the high energy turnover trial compared to the low energy turnover trial (low energy turnover: 90.9± 13.8 $pg.ml^{-1}$, high energy turnover: 73.5 ± 10.9 $pg.ml^{-1}$, $p < 0.05$). Two-way analysis of variance revealed significantly lower acylated ghrelin concentrations in the high energy turnover trial at 0, 2 and 4 hours but not 6 hours (Figure 7.4). Calculation of the incremental AUC revealed no differences between trials for either the total ghrelin (low energy turnover: 0.01 ± 0.08 $ng.ml^{-1}$, high energy turnover: -0.01 ± 0.17 $ng.ml^{-1}$, $p > 0.05$) or acylated ghrelin (low energy turnover: -8.7 ± 6.3 $pg.ml^{-1}$, high energy turnover: -3.3 ± 5.5 $pg.ml^{-1}$, $p > 0.05$) postprandial responses.

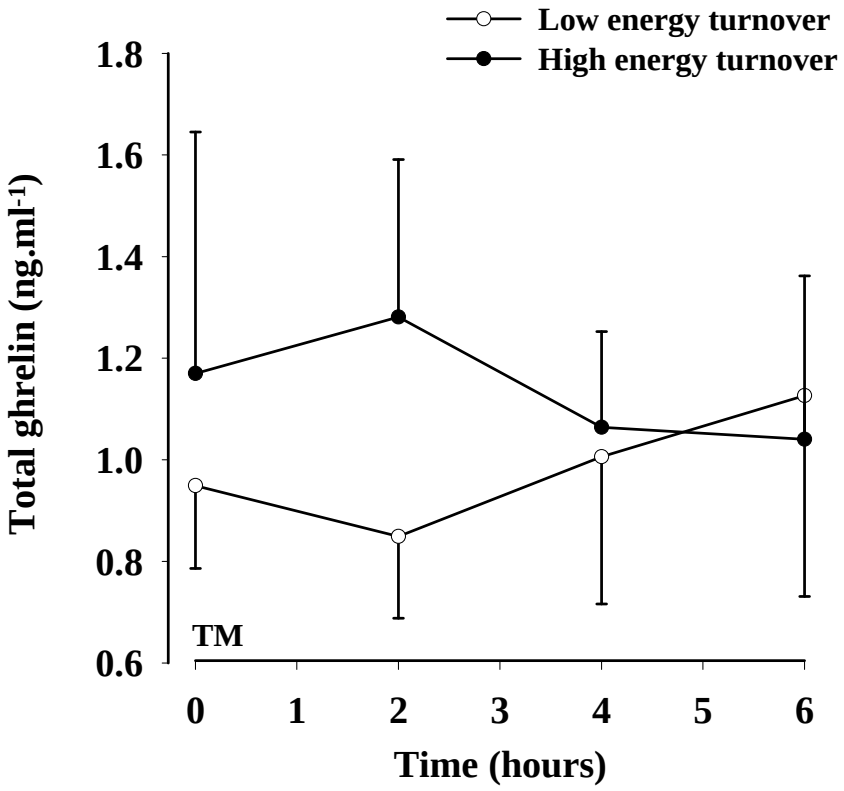


Figure 7.3 Plasma total ghrelin response during the low energy turnover (o) and high energy turnover (●) trials. TM indicates the time at which the buffet meal was provided.

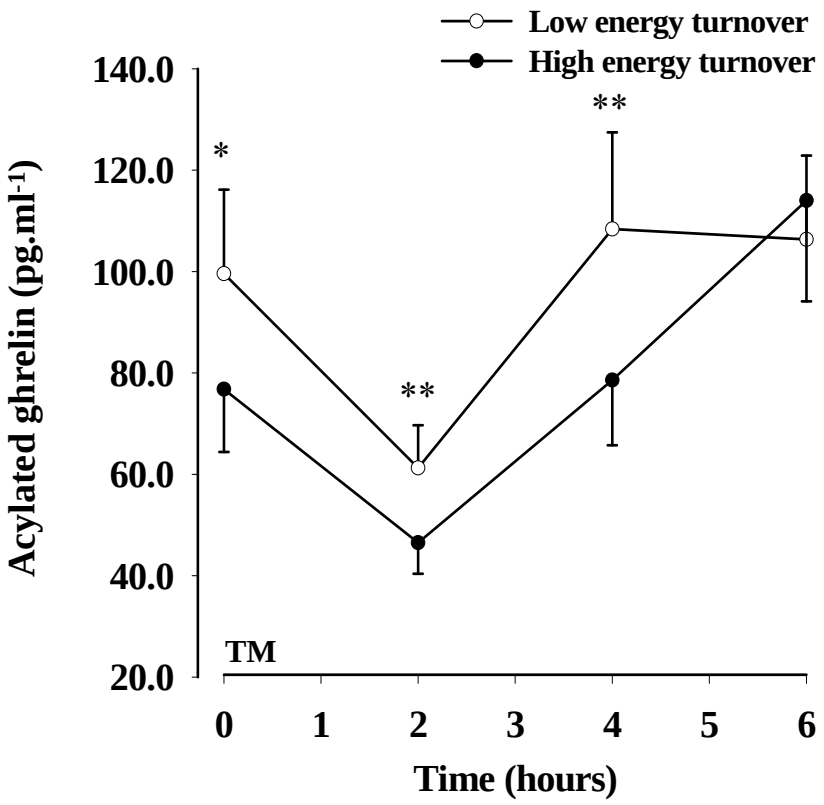


Figure 7.4 Plasma acylated ghrelin response during the low energy turnover (o) and high energy turnover (●) trials. TM indicates the time at which the buffet meal was provided. ** $p < 0.01$. * $p < 0.05$.

Time-averaged appetite ratings are shown in **Figure 7.5**. There were no differences between the low and high energy turnover trials in ratings of hunger (low energy turnover: 53.2 ± 4.1 mm, high energy turnover: 50.3 ± 4.8 mm, $p > 0.05$), satiety (low energy turnover: 39.6 ± 3.4 mm, high energy turnover: 44.2 ± 4.1 mm, $p > 0.05$), fullness (low energy turnover: 37.5 ± 4.0 mm, high energy turnover: 39.9 ± 4.2 mm, $p > 0.05$), PFC (low energy turnover: 61.1 ± 3.1 mm, high energy turnover: 58.1 ± 4.4 mm, $p > 0.05$) or desire (low energy turnover: 54.9 ± 3.8 mm, high energy turnover: 50.8 ± 4.7 mm, $p > 0.05$). Two-way analysis of variance did however reveal significant trial by time interactions, shown in **Figure 7.6**. One hour after the test meal, hunger scores were 27% lower in the high energy turnover trial compared to the low energy turnover trial ($p < 0.05$). At 30 minutes and 1 hour, satiety scores were 14% ($p < 0.05$) and 17% ($p < 0.05$) higher, respectively, in the high energy turnover trial and at 30 minutes and 4 hours, fullness scores were 10% ($p < 0.05$) and 28% ($p < 0.05$) higher, respectively, in the high energy turnover trial compared to the low energy turnover trial. Thirty minutes after the test meal,

PFC scores were 35% lower in the high energy turnover trial ($p < 0.001$). Desire did not differ between trials until 4 hours where desire was 14% lower in the high energy turnover trial compared to the low energy turnover trial ($p < 0.05$).

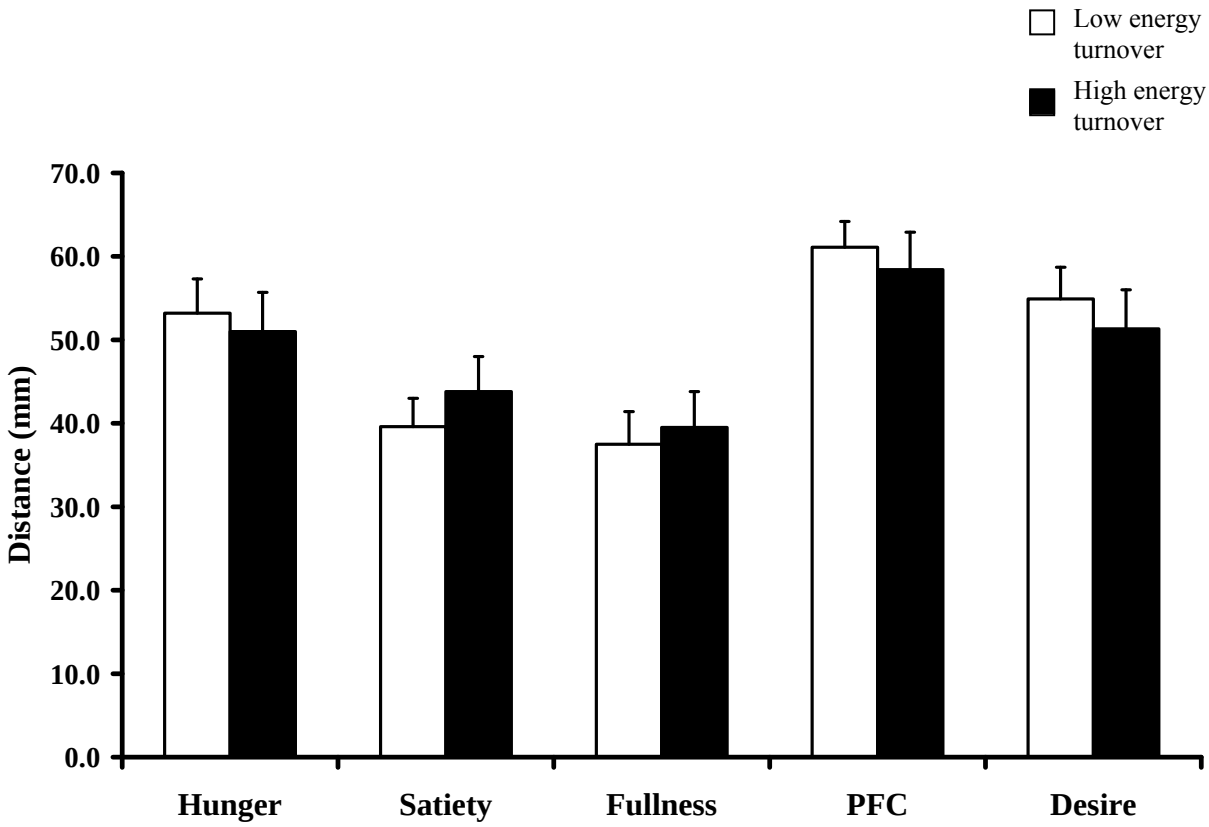


Figure 7.5 Time-averaged mean postprandial appetite ratings.

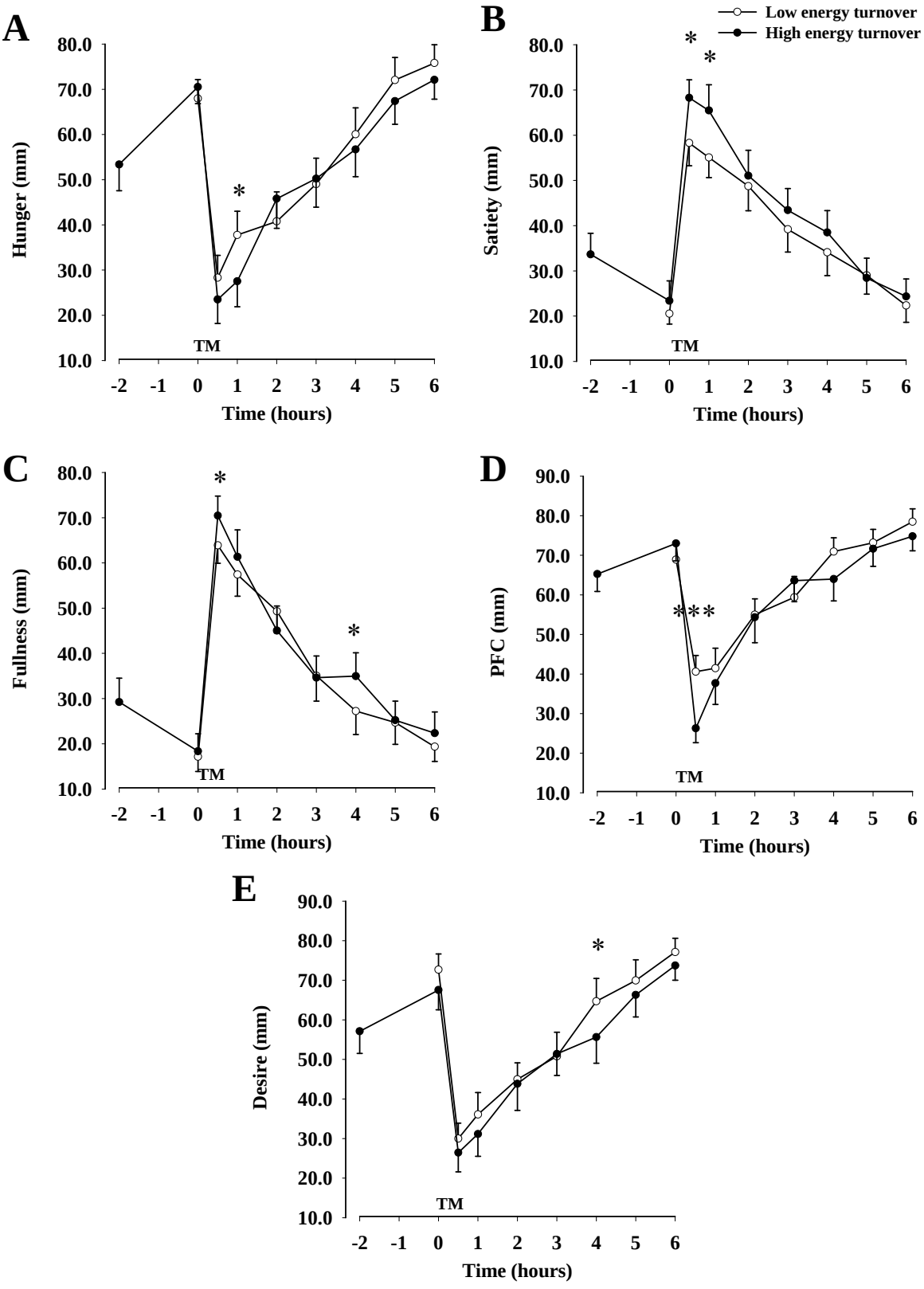


Figure 7.6 Hunger (A), satiety (B), fullness (C), prospective food consumption (PFC, D) and desire (E) ratings during the low energy turnover (o) and high energy turnover (●) trials. TM indicates the time at which the test meal was provided. *** $p < 0.001$, * $p < 0.05$, for differences between trials.

7.3.5 Relationships between variables

7.3.5.1 Total ghrelin, acylated ghrelin and subjective ratings of appetite

Neither fasting nor postprandial total ghrelin or acylated ghrelin concentrations correlated with age, BMI or waist circumference. No correlations were observed between fasting total ghrelin or acylated ghrelin concentrations and any of satiety, fullness, PFC or desire scores. Fasting total ghrelin concentrations correlated with fasting hunger in low energy turnover ($r = 0.67$, $p < 0.05$) but not high energy turnover and similarly, fasting acylated ghrelin concentrations correlated with fasting hunger ($r = 0.70$, $p < 0.05$) in low but not high energy turnover.

Postprandial total ghrelin and acylated ghrelin were both inversely correlated with postprandial glucose concentrations (total ghrelin *vs.* glucose: $r = -0.54$, $p < 0.05$, acylated ghrelin *vs.* glucose: $r = -0.53$, $p < 0.05$) (**Figure 7.7**). There were no correlations between total ghrelin or acylated ghrelin and any of hunger, satiety, fullness, PFC or desire scores during the postprandial period. Exercise-induced changes in postprandial acylated ghrelin responses were however inversely correlated with exercise-induced changes in satiety ($r = -0.64$, $p < 0.05$) (**Figure 7.8**), indicating that lower satiety may be mediated by higher postprandial concentrations of acylated ghrelin. In addition, exercise-induced changes in postprandial hunger were inversely correlated with changes in postprandial glucose concentrations ($r = -0.61$, $p < 0.05$) (**Figure 7.9**), suggesting that greater hunger was coupled with smaller increases in glucose.

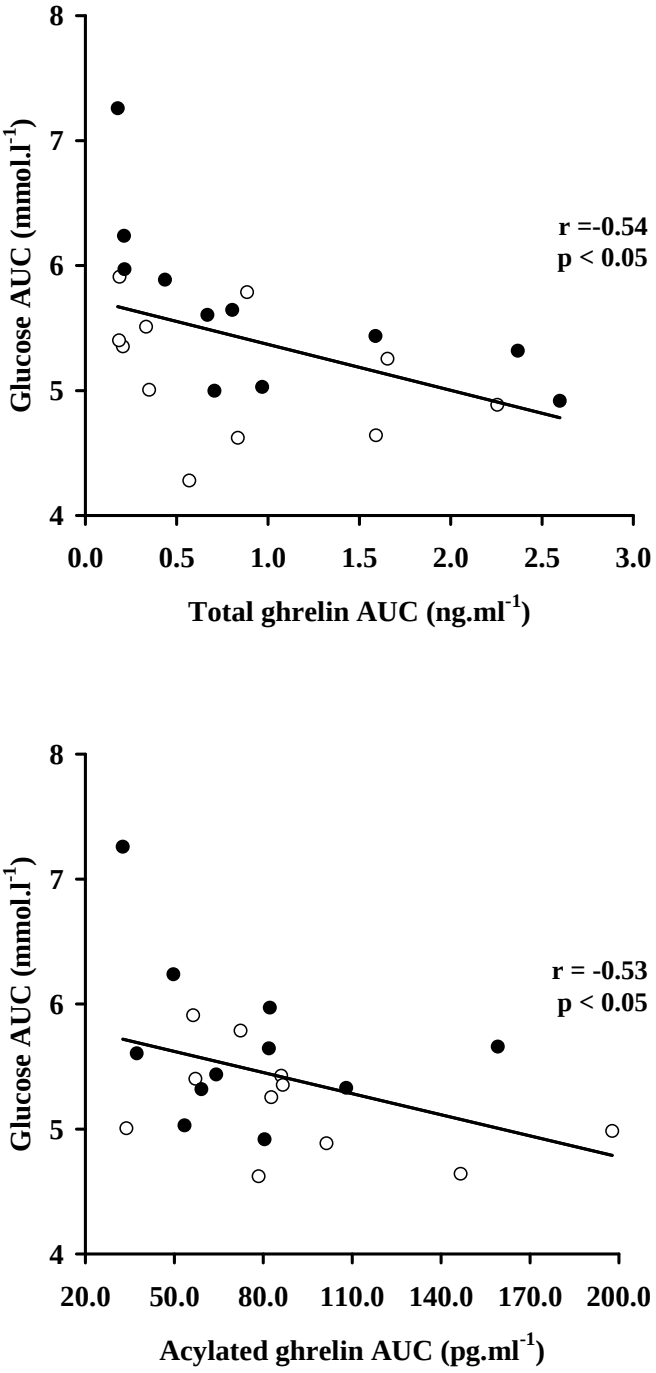


Figure 7.7 The relationship between postprandial total ghrelin and postprandial glucose concentrations (top) and between postprandial acylated ghrelin and postprandial glucose concentrations (bottom) in the high energy (●) and low energy (O) turnover trials.

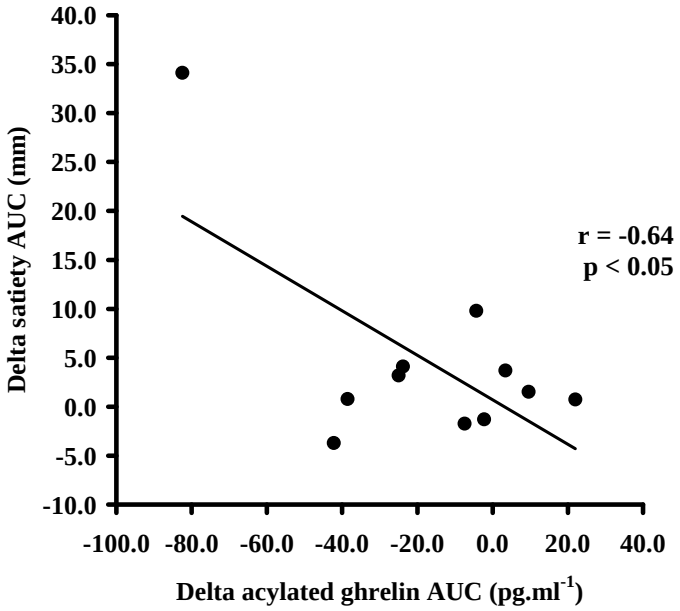


Figure 7.8 The relationship between exercise-induced changes in postprandial acylated ghrelin concentrations and postprandial ratings of satiety.

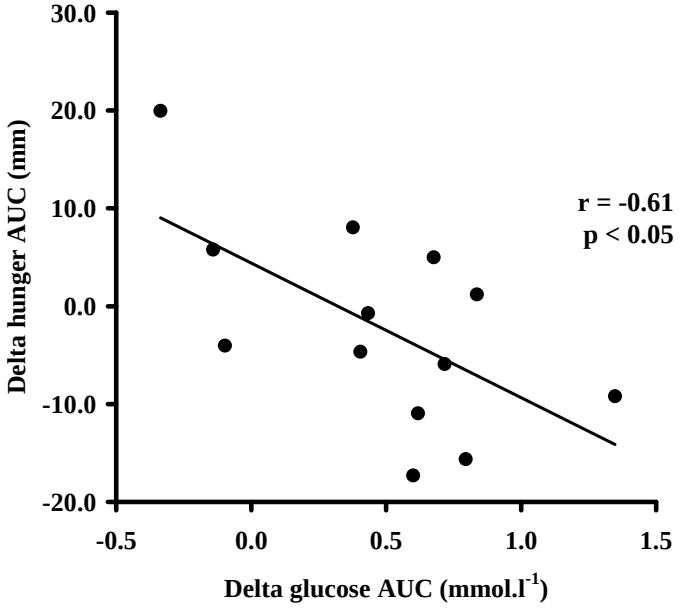


Figure 7.9 The relationship between exercise-induced changes in postprandial glucose concentrations and postprandial ratings of hunger.

7.3.5.2 Energy and macronutrient intakes

There were no correlations between energy or macronutrient intake at the buffet meal and glucose or insulin concentrations in either the low or high energy turnover trials. Energy intake was correlated with exercise-induced changes in total ghrelin although surprisingly the correlation was negative ($r = -0.71$, $p < 0.05$) **(Figure 7.10)**, suggesting that less food was eaten when there was a greater increase in total ghrelin concentrations. Similar relationships were also observed between total ghrelin and protein intake ($r = -0.79$, $p < 0.01$) and fat intake ($r = -0.68$, $p < 0.05$) and a tendency was also seen in carbohydrate intake ($r = -0.57$, $p = 0.070$). Exercise-induced changes in acylated ghrelin were not correlated with energy or macronutrient intakes. A positive association was observed between energy intake at the buffet meal and postprandial carbohydrate oxidation ($r = 0.42$, $p < 0.05$). Carbohydrate intake at the buffet meal was also correlated with postprandial carbohydrate oxidation ($r = 0.52$, $p < 0.01$) and inversely correlated with postprandial fat oxidation ($r = -0.42$, $p < 0.05$) (**Figure 7.11**).

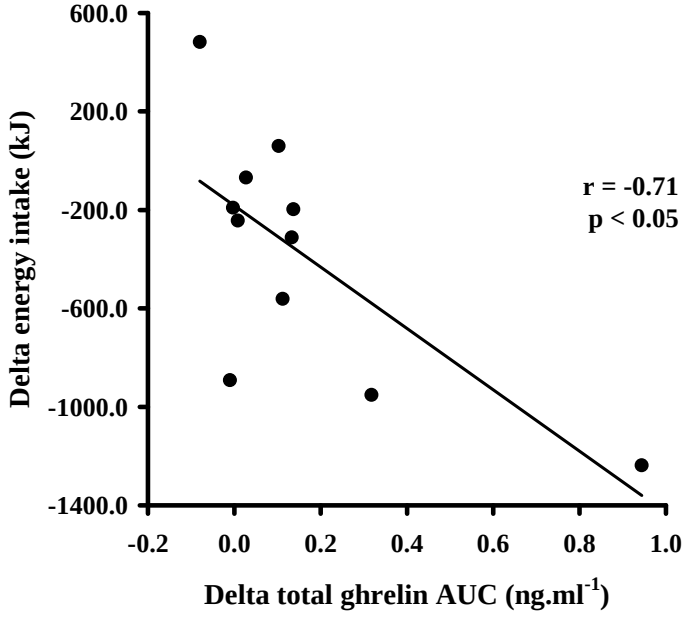


Figure 7.10 The relationship between exercise-induced changes in postprandial total ghrelin concentrations and changes in energy intake at the buffet meal.

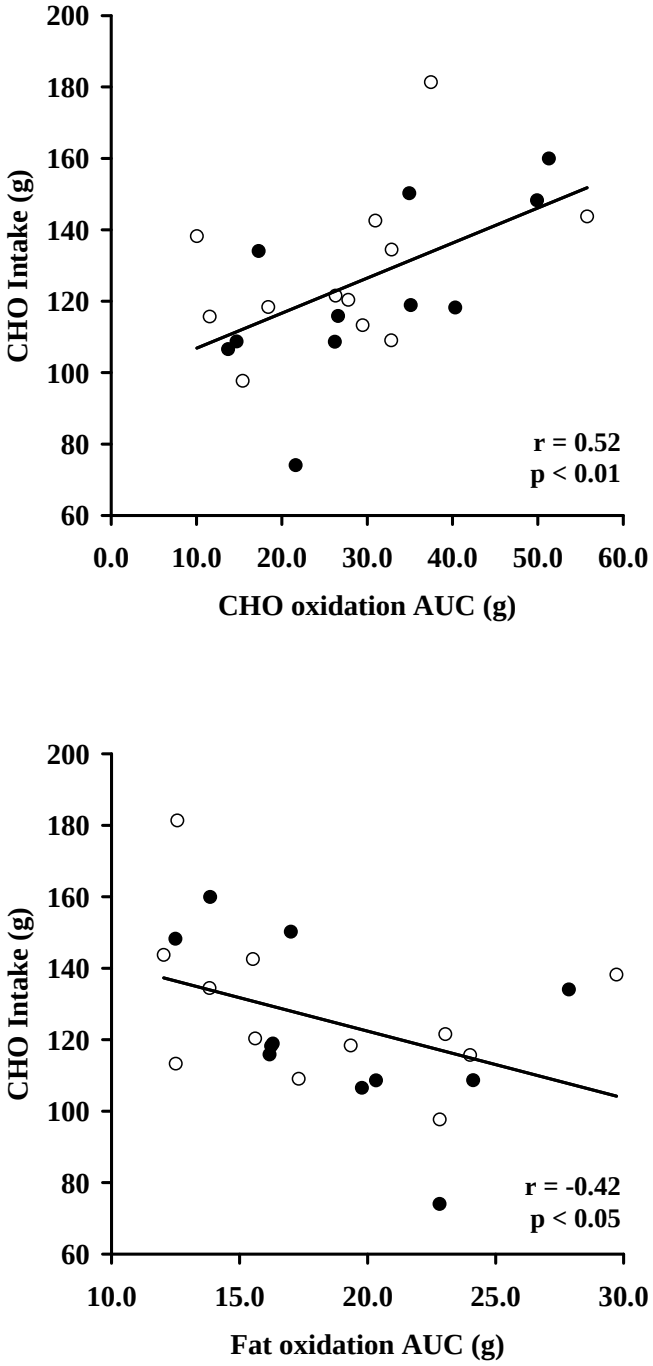


Figure 7.11 The relationship between postprandial carbohydrate (CHO) oxidation and carbohydrate intake at the buffet meal (top) and between postprandial fat oxidation and carbohydrate intake at the buffet meal (bottom) in the high energy (●) and low energy (O) turnover trials.

7.4 Discussion

The present study was designed to compare differences in appetite regulation and feeding behaviour between states of energy balance with either high or low energy turnover. Results showed no trial effect on energy and macronutrient intake but during the high energy turnover trial, fat balance was attenuated and at the end of each trial, on completion of the buffet meal, both energy balance and fat balance were significantly lower in the high energy turnover trial compared to the low energy turnover trial. The total ghrelin response did not differ between trials, however fasting and postprandial acylated ghrelin concentrations were significantly lower in the high energy turnover trial compared to the low energy turnover trial, a response which may, at least in part, be mediated by changes in postprandial glucose concentrations. Finally, the high energy turnover trial suppressed hunger and PFC and increased satiety and fullness in the short-term compared to the low energy turnover trial.

Enhanced appetite and weight control in people who regularly exercise (King *et al.*, 1997b;Wareham *et al.*, 2005) could be mediated by changes in appetite regulation and feeding behaviour. Although the present study found no significant trial effect in any of the appetite ratings, trial by time interactions did reveal that during the short-term postprandial period (up to one hour), exercise-induced high energy turnover suppressed hunger and PFC and increased satiety and fullness. These findings are consistent with data from others also reporting lower hunger (Burns *et al.*, 2007;King *et al.*, 1994;Westerterp-Plantenga *et al.*, 1997a) and increased satiety (Tsofliou *et al.*, 2003) after acute exercise. A novel finding of this study is, however, that the exercise-induced effect on appetite remained even when the associated energy deficit was replaced. It does though remain apparent that any modification of appetite ratings following exercise is a relatively short lived phenomenon. The glucostatic theory was developed as a potential mechanism for the relationship between glucose and appetite (Mayer, 1953). Studies show a decline in plasma glucose which precedes meal initiation (Anderson & Woodend, 2003), whilst glucose concentrations are also associated with satiety (Raben *et al.*, 1996) and energy intake (Flint *et al.*, 2006). Insulin has been implicated in the regulation and control of appetite and is related to a suppression in hunger (Flint *et al.*, 2007), increase in satiety and fullness (Flint *et al.*, 2006;Flint *et al.*, 2007) and lower energy intake (Flint *et al.*, 2007). The present study found no relationship between glucose or insulin and any of the subjective appetite ratings or energy intake in either of the two trials. Exercise-induced changes in postprandial glucose concentrations were, however, inversely correlated with postprandial changes in hunger, suggesting that larger glucose responses suppressed

hunger, which could be taken to reflect increased satiety, a finding which is consistent with others (Mayer, 1953;Raben *et al.*, 1996). It is not clear why no relationship between glucose and energy intake was evident in the present study, although use of a fixed time point for the buffet meal, which may have preceded any significant decline in glucose concentrations, could have masked such an association (Flint *et al.*, 2007). The study of overweight women may also explain the absence of any significant correlations as the relationship between glucose or insulin, appetite and energy intake may be disrupted in the overweight and obese (Flint *et al.*, 2007).

Ghrelin remains a hormone of interest due to its role in stimulating appetite and food intake (Druce *et al.*, 2005;Wren *et al.*, 2001). However, studies have consistently failed to show any effect of acute exercise on total ghrelin concentrations (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a). Chapter 5 extended these findings revealing that exercise, with the associated energy deficit replaced, also failed to attenuate fasting and postprandial total ghrelin concentrations. It is perhaps not surprising therefore, that the present study found no difference in total ghrelin between high and low energy turnover, or that during the postprandial period, ghrelin was not associated with subjective ratings of appetite. Interestingly the present study did observe a significant reduction in fasting and postprandial acylated ghrelin concentrations in high energy turnover. Acylated ghrelin is the active component of ghrelin involved in appetite regulation (Broom *et al.*, 2007;Mackelvie *et al.*, 2007), and thus the ratio of acylated to de-acylated ghrelin is important. To the author's knowledge only two studies have investigated the effects of exercise on acylated ghrelin (Broom *et al.*, 2007;Mackelvie *et al.*, 2007) and consistent with the data presented here, both reported acylated ghrelin to be lower following acute exercise. The present study also shows that exercise, even when the associated energy deficit is replaced and energy balance maintained, lowers the postprandial acylated ghrelin response. In contrast to Broom *et al*. (Broom *et al.*, 2007) the present study found no association between postprandial acylated ghrelin and hunger ratings although exercise-induced changes in postprandial acylated ghrelin and postprandial satiety were inversely correlated. The exciting findings of this study and those of others (Broom *et al.*, 2007;Mackelvie *et al.*, 2007) suggest that although total ghrelin *per se* may not respond to exercise or modulate appetite, exercise may still be a successful strategy for mediating appetite via its effect on acylated ghrelin. This may be a mechanism by which regular exercisers experience better weight control and appetite regulation (King *et al.*, 1997b;Wareham *et al.*, 2005). It is difficult to determine how or why a single session of

exercise attenuates acylated ghrelin but not total ghrelin concentrations although a more rapid response of acylated ghrelin to carbohydrate or glucose availability has been suggested (Hosoda *et al.*, 2004;Tannous dit *et al.*, 2006) Therefore, in the present study, additional carbohydrate provided in the high energy turnover test meal may have stimulated a greater attenuation in acylated ghrelin. Future research will undoubtedly provide greater insight into how exercise affects acylated ghrelin, the potential mechanisms for such an effect, and furthermore, what the implications of this may be for appetite and body weight regulation.

Directly measuring energy and macronutrient intake is used to determine the effects of exercise on appetite and subsequent food intake. Studies using such an approach have however provided equivocal findings; some report an increase (Stubbs *et al.*, 2002b) whilst others show no change in energy intake (George & Morganstein, 2003;Hubert *et al.*, 1998;Imbeault *et al.*, 1997;King *et al.*, 1997a) following exercise. Equally unclear is the effect of exercise on macronutrient preferences with studies showing increased fat and carbohydrate intake (Pomerleau *et al.*, 2004;Stubbs *et al.*, 2004;Westerterp-Plantenga *et al.*, 1997a) and other studies reporting no effect (Hubert *et al.*, 1998;King *et al.*, 1997a). Findings presented here agree with many others, showing no difference in energy intake between high or low energy turnover. There were also no differences in macronutrient preferences although a tendency to consume more carbohydrate during the low energy turnover trial was observed. Interestingly, carbohydrate intake during the buffet meal strongly correlated with postprandial carbohydrate oxidation suggesting that more carbohydrate was consumed in response to a greater amount of carbohydrate being oxidised. This may be a regulatory mechanism designed to replenish fuel sources that are depleted during exercise (Bellisle, 1999). Total energy, protein, fat and to a lesser extent carbohydrate intakes were all correlated with exercise-induced changes in total ghrelin, but not acylated ghrelin. Surprisingly however, these relationships were negative suggesting that larger ghrelin responses suppressed energy and macronutrient intake. This is the opposite of both what was expected and what has previously been reported (Druce *et al.*, 2005;Wren *et al.*, 2001), although it does show some similarities with data reported in Chapter 5, where total ghrelin was inversely correlated with hunger and positively correlated with satiety. A possible hypothesis, which may warrant future investigation, is that replacing the energy deficit associated with exercise somehow reverses the normal orexigenic properties of ghrelin in people who are overweight and obese.

Fat balance is known to closely reflect energy balance, both of which have important implications for future weight gain and obesity (Flatt *et al.*, 1985). Calculation of cumulative energy and energy substrate balances in the present study, allowed for changes in energy substrate oxidation, during and following exercise, to be considered in relation to energy, fat and carbohydrate intake. Cumulative energy balance was not different during the high and low energy turnover trials, however after consumption of the buffet meal, energy balance was lower in the high energy turnover trial, suggesting that energy intake during the meal was lower. Although analyses revealed no significant differences when energy intakes were directly compared, there was a tendency for intake to be lower in the high energy turnover trial. The findings of the present study agree with those of Stubbs *et al*. (Stubbs *et al.*, 2002a;Stubbs *et al.*, 2002b) who reported lower cumulative energy balance after exercise, and furthermore, they suggest that exercise even with energy replacement attenuates subsequent energy balance. Cumulative fat balance was lower during the high energy turnover trial and remained lower after completion of the buffet meal, suggesting that although postprandial total fat oxidation did not differ significantly between trials during the postprandial observation period, the elevated rate of fat oxidation during exercise, as previously reported (Goodpaster *et al.*, 2002;Martin, III, 1996), may attenuate subsequent fat balance, even if energy balance is maintained. Considering that more positive fat balances are associated with future weight gain (Flatt *et al.*, 1985), the finding of lower fat balance in the high energy turnover trial has potentially important implications for future weight management. Interestingly, although cumulative carbohydrate balance was elevated during the high energy turnover trial prior to the buffet meal, carbohydrate balance was no longer different on completion of the two trials after the buffet meal was eaten. This might be explained by the difference in carbohydrate intake at the buffet meal, which although not significant, did tend to be lower in the high energy turnover trial compared to low energy turnover trial (high energy turnover: 118.7 ± 6.7 g *vs.* low energy turnover: 128.8 ± 5.9 g, $p = 0.08$). Considered together, these data suggest that a state of energy balance with high energy turnover promotes changes in energy and fat balances which may in turn facilitate weight maintenance. This may help to explain why regular exercisers more effectively resist future weight gain and maintain stable body weights (Wareham *et al.*, 2005).

Currently, there is no satisfactory explanation for why some report changes in energy and macronutrient intake following exercise whilst others report no such effect. Subject population may explain some of the variation (Stubbs *et al.*, 2002a), and considering that, with regard to appetite and food intake, men and women (Blundell *et al.*, 2003) and also

overweight and normal weight populations (King *et al.*, 1997b) respond differently to exercise, this is likely to be important. A strength of the current study is that subjects were not aware that that their food intake was being measured, and rather believed that the study was investigating how exercise effects markers of food palatability. Such an approach was used to avoid the possibility of the subjects' own personal beliefs and perceptions of how much food is acceptable to eat influencing food intake (Bock & Kanerek, 1995). Conversely, a potential limitation of the present study is that food intake was not measured during a follow up period. Compensatory changes in food intake may not occur immediately but rather, gradually, over a period of days or weeks (Stubbs *et al.*, 2002b). Measurement of food intake within the laboratory may also limit the current findings as within such an environment, social facilitation, food cost, food type etc, factors which may all mediate feeding behaviour, are tightly controlled (De Castro, 2000). It would be interesting to observe whether a state of energy balance with either high or low energy turnover modulates energy and macronutrient intake when an individual is allowed to eat in their more typical, every day environment.

In summary, data from the present study suggest that a state of energy balance with high or low energy turnover is different with regard to appetite regulation and feeding behaviour. Lower cumulative fat balances in high energy turnover will have important implications for the prevention of future weight gain. A short term suppression of hunger and PFC and an increase in satiety and fullness, in addition to lower postprandial acylated ghrelin concentrations may contribute to enhanced appetite regulation during a state of energy balance with high energy turnover. Together, these findings provide insight into how regular exercisers, a population who are better able to prevent weight gain, may be metabolically and behaviourally different to their sedentary peers. Future research investigating whether the current findings can be extended to populations such as overweight and obese men, patients with type 2 diabetes and adolescents and also to an environment outside of the laboratory, will prove interesting and hopefully further elucidate some of the mechanisms underlying these effects.

CHAPTER 8
GENERAL DISCUSSION

8.1 Experimental chapter summaries

The aim of Chapter 3 was to determine the extent to which exercise-induced changes in postprandial metabolism were mediated by the associated energy deficit, with a particular emphasis on postprandial lipid metabolism, insulin and glucose metabolism and energy substrate oxidation. The findings suggested that exercise, even when the associated energy deficit was replaced significantly attenuated insulin concentrations and increased whole body fat oxidation. Both effects were, however, augmented when the exercise-induced energy deficit was maintained, and an energy deficit was required to lower postprandial TG concentrations. The attenuation in postprandial lipaemia observed the day following exercise was associated with changes in whole body and hepatic fat oxidation, suggesting that increased fat oxidation may have mediated, at least in part, lower TG concentrations.

Chapter 4 aimed to investigate the effects of exercise, with and without energy replacement, on fasting and postprandial radial PWV. A second aim was to investigate whether acute exercise altered plasma ADMA concentrations, and to determine whether changes in PWV were mediated by changes in ADMA. The main finding of Chapter 4 was that exercise lowered fasting and postprandial radial PWV independently of the exercise-induced energy deficit. The same exercise sessions did not, however, influence plasma ADMA concentrations and thus ADMA is unlikely to have mediated any changes in PWV.

Chapter 5 aimed to determine the effects of exercise, with and without energy replacement, on appetite control and appetite hormones. A second aim was to investigate whether exercise-induced changes in plasma total ghrelin and leptin mediated any subsequent changes in appetite. Exercise had no effect on any of the subjective ratings of appetite including hunger, satiety, fullness, prospective food consumption (PFC) and desire. Exercise also did not influence fasting or postprandial total ghrelin concentrations. However, fasting and postprandial leptin concentrations were attenuated following exercise with and exercise without energy replacement. Changes in plasma leptin may have been associated with changes in postprandial glucose concentrations and energy substrate oxidation.

The aim of Chapter 6 was to compare the immediate effects of a state of energy balance with either high or low energy turnover on postprandial metabolism with a particular focus on postprandial lipaemia, insulinaemia, energy substrate oxidation and PWV. The postprandial TG response was not different between the two energy balance states, however postprandial insulin and glucose concentrations were elevated in the high energy turnover trial. Postprandial energy expenditure and fat oxidation were elevated during the initial hours following the test meal in the high energy turnover trial. Neither postprandial radial nor femoral PWV differed between the high energy turnover and low energy turnover trials.

Chapter 7 compared appetite regulation and feeding behaviour in a state of energy balance with either high or low energy turnover. A second aim was to compare the effect of energy balance coupled with high or low energy turnover on total ghrelin and acylated ghrelin concentrations. Hunger and PFC were both suppressed and satiety and fullness were increased in the high energy turnover trial. Energy and macronutrient intake did not differ, but at the end of the day, after eating a meal, energy balance and fat balance were significantly lower in the high energy turnover trial. Postprandial total ghrelin concentrations did not differ between trials, however postprandial acylated ghrelin concentrations were significantly lower in the high energy turnover trial, an effect which appeared to be associated with changes in postprandial glucose concentrations.

Considerable work has already been published investigating the effects of exercise with an energy deficit on postprandial metabolism, appetite regulation and feeding behaviour. The aim of this thesis was to determine what the role for an energy balance state was in mediating such exercise-induced changes. Therefore, the following discussion will primarily focus on the observed responses to exercise coupled with a state of energy balance and compare the state of energy balance coupled with either high (exercise) or low (rest) energy turnover. The collection of postprandial data both immediately after exercise and the following day enables the author to consider the response to exercise during the subsequent 30 hours or so, however, the reader must take into consideration the fact that the immediate response to exercise was determined in women whilst the delayed response was observed in men, and that some gender differences in the responses might exist**.**

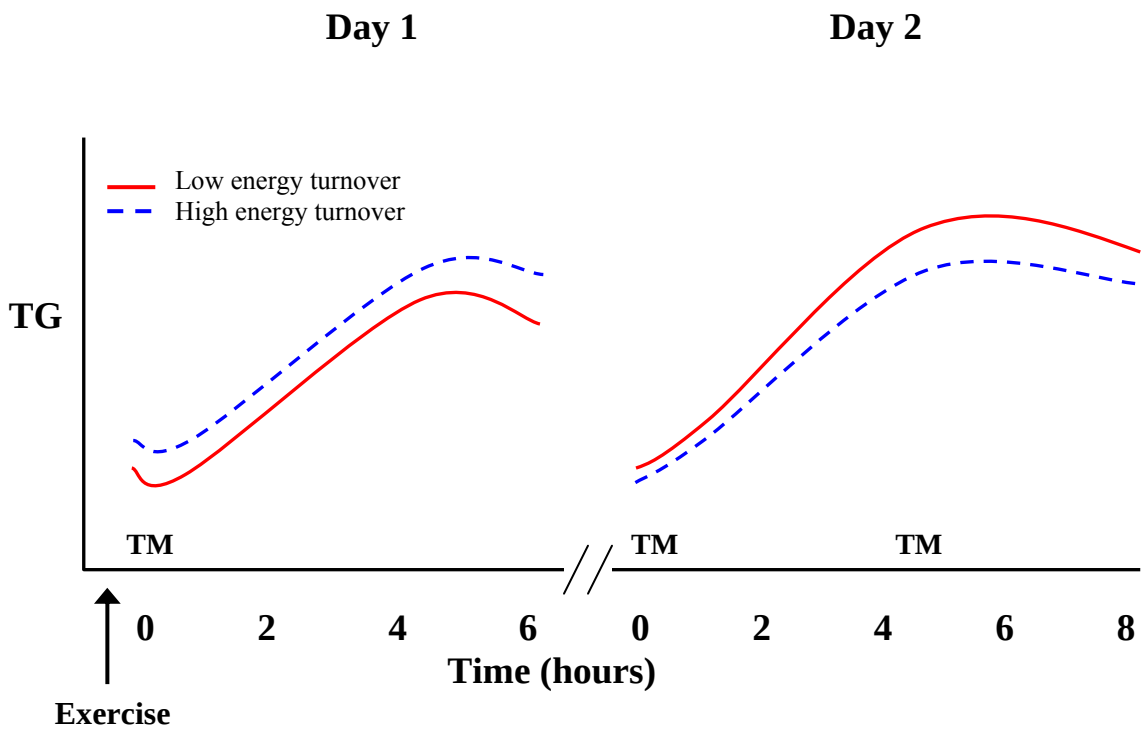


Figure 8.1 Postprandial TG responses in a state of energy balance with high and low energy turnover.

During day one, there was no significant effect of energy turnover on postprandial TG concentrations (**Figure 8.1**), an effect not unexpected due to previous observations that any effect of exercise with an energy deficit appears to occur after a delay of some hours (Malkova & Gill, 2006). On day two, the TG response was significantly lower following exercise with an energy deficit, findings consistent with those from others (Aldred *et al.*, 1994;Gill & Hardman, 2000;Tsetsonis *et al.*, 1997) but a novel finding of this study was that maintaining energy balance by eliminating the associated energy deficit attenuated such an effect with the TG response just 6% lower in the high energy turnover trial. The lower TG response following exercise might have been mediated by a reduction in the hepatic secretion of VLDL particles; a rise in 3-OHB concentrations on day two perhaps suggests that fatty acids arriving at the liver were directed towards oxidation rather than secretion within VLDL. These findings are consistent with two earlier studies also reporting 3-OHB concentrations to be elevated post-exercise (Gill *et al.*, 2006a;Gill *et al.*, 2001a). Changes in 3-OHB did, however, only explain 23% of the variation in the changes in TG and thus it is likely that changes in other metabolic pathways contributed to the attenuated TG response. In fact, the lower TG following exercise might have been a consequence of lower glycogen stores, stores which are depleted in both the liver and

skeletal muscle following prolonged exercise (Casey *et al.*, 2000;Kiens & Richter, 1998). Whilst the body strives to replenish these glycogen stores it switches to the use of lipids, i.e. TG, as a fuel source to spare glucose for glycogen synthesis. Indeed, Kiens and Richter have reported intramuscular TG to be reduced by 20% when measured 18 hours post exercise (Kiens & Richter, 1998). It is possible, therefore, that the attenuation of any lower TG response in energy balance with high energy turnover, may be explained by the augmented replenishment of glycogen stores, thus reducing the need to use lipids as a fuel source.

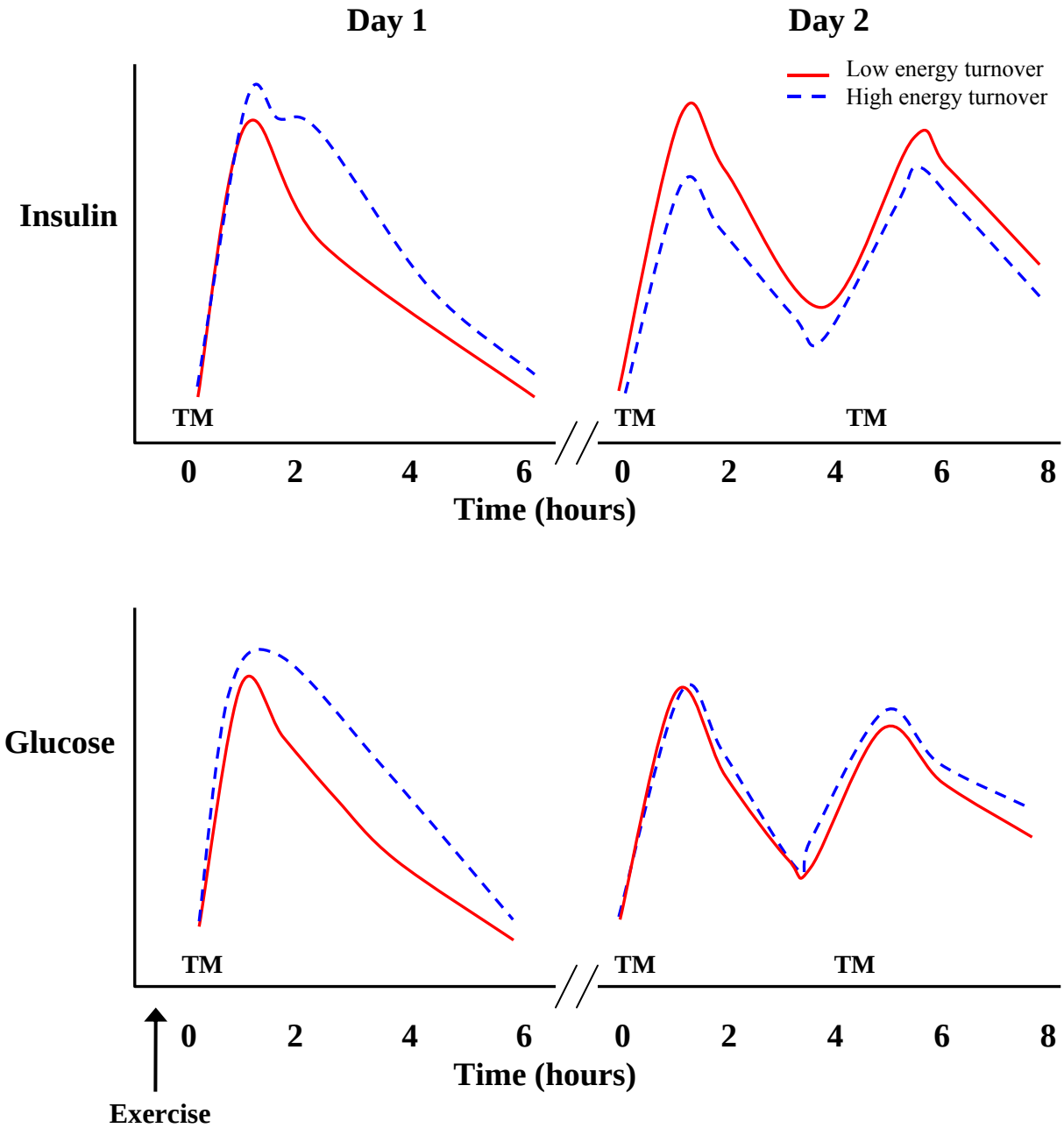


Figure 8.2 Postprandial insulin (top) and glucose (bottom) responses in a state of energy balance with high and low energy turnover.

The glucose response on day one was unexpectedly higher with high energy turnover compared to low (**Figure 8.2**). Although a similar post-exercise rise in glucose has been reported recently, those authors made no attempt to explain why such a response occurred (Broom *et al.*, 2007). In the present study, however, it appears that higher glucose concentrations were a consequence of elevated fatty acid concentrations, which via the glucose-fatty acid cycle, prevent the uptake and oxidation of glucose within tissues (Randle *et al.*, 1963) causing their concentration to build up within the blood. This was however a relatively transient effect and from five hours on day one through to completion of day two, no significant difference in glucose concentrations was observed, findings consistent with those of others showing no change in glucose measured the day following a single session of exercise with an energy deficit (Gill *et al.*, 2006a;Tsetsonis & Hardman, 1996). To the author's knowledge, no other study has reported elevated insulin concentrations during the immediate post-exercise period, and subsequently it is suggested that the higher insulin concentrations on day one are a secondary effect of higher glucose. Any effect of exercise did appear to occur after some delay, as on day two, the plasma insulin response was lower and insulin sensitivity appeared to be enhanced in a state of energy balance with high energy turnover (Figure 8.2), although maintaining an energy deficit did augment such an effect. Although no direct evidence is available, it is possible that increased availability of GLUT-4 transporters post-exercise may have enhanced insulin mediated glucose uptake (Kennedy *et al.*, 1999;Kraniou *et al.*, 2006). Again however it is also possible that depleted glycogen stores might have facilitated enhanced insulin sensitivity (Wojtaszewski *et al.*, 2002). The accentuated insulin response when energy balance was maintained might therefore be explained by the extra food provided in the energy replacement trial (Cartee *et al.*, 1989) and a greater degree of glycogen repletion within tissues.

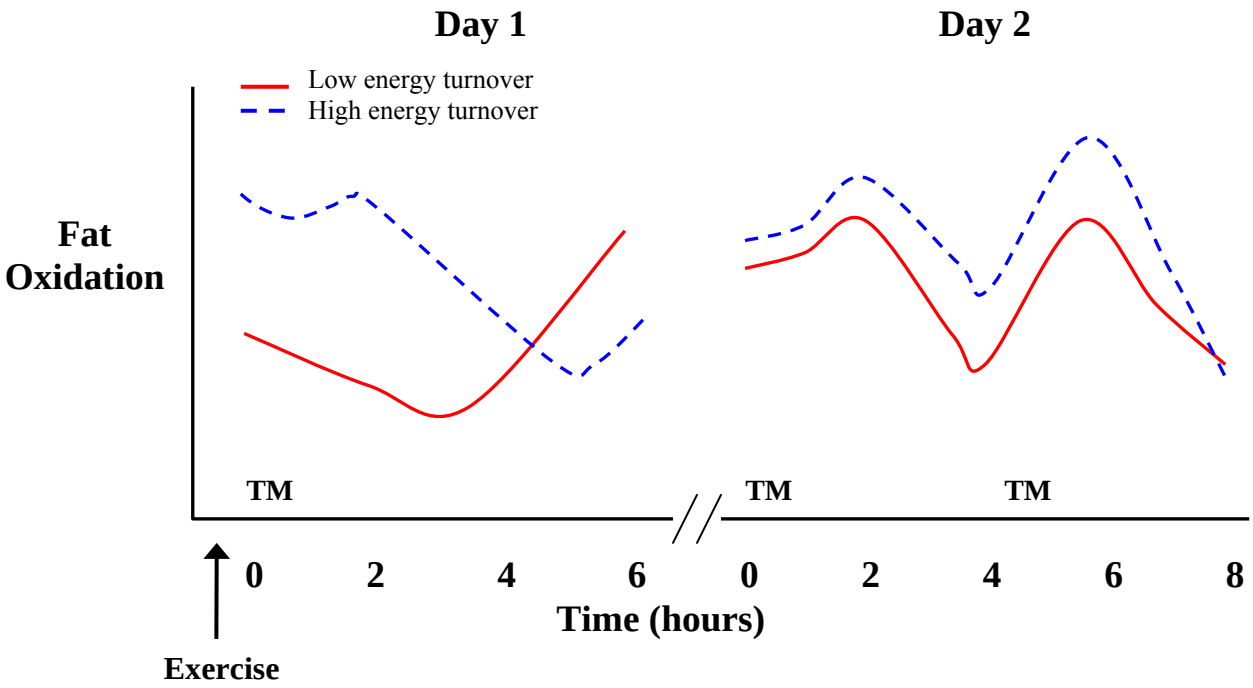


Figure 8.3 Postprandial fat oxidation in a state of energy balance with high and low energy turnover**.**

In the present study, whole body fat oxidation was elevated for some three hours on day 1 after exercise (**Figure 8.3**) an effect which has previously been reported (Votruba *et al.*, 2002;Votruba *et al.*, 2005), although a novel finding here was that even in a state of energy balance, the high energy turnover increased fat oxidation. Elevated concentrations of circulating fatty acids will likely have mediated the higher fat oxidation although an increase of 3-OHB concentrations during the post-exercise period, which appeared to be an effect independent of the rise in plasma fatty acids, suggest that greater hepatic fat oxidation may also be important in the initial hours post-exercise. On day one, however, a decline in fat oxidation was observed around fours hours postprandially. This coincided with a rise in carbohydrate oxidation and perhaps suggests that during this time, elevated glucose concentrations that occurred with a high energy turnover induced greater glucose oxidation at the expense of fat utilisation (Frayn, 1998). On day two however, fat oxidation remained elevated in a state of energy balance with high energy turnover compared to low energy turnover, although maintaining the exercise-induced energy deficit augmented such an effect. Greater fat oxidation observed some hours after exercise is likely to reflect the body's reliance upon lipids as a fuel source to spare glucose for glycogen repletion and would therefore explain why the increased fat oxidation was of a

greater magnitude following exercise with an energy deficit, which would have promoted greater glycogen depletion within tissues. Unfortunately, it is not possible to comment on the exact source of this greater fat oxidation, and therefore the above mechanisms are speculative. The use of labelled fats in future studies may, however, provide more accurate insight into the proportion of endogenous and exogenous fat that is oxidised following exercise in a state of energy balance and the implications of such changes for other metabolic pathways.

An interesting finding from Chapter 7 was that fat balance, both prior to and on completion of a buffet meal, was significantly lower in energy balance with high energy turnover, despite the extra amount of fat consumed in the test meal. This suggested that exercise-induced changes in fat oxidation, observed during the hours immediately following exercise, were substantial enough to counteract the higher fat intake and thus attenuate fat balance. As fat balance closely reflects the state of energy balance, due to the inability of fat oxidation to up regulate itself with excess fat availability (Flatt, 1995), the findings of greater fat oxidation and lower fat balances in a state of energy balance with high energy turnover has exciting implications for the future control, management and treatment of obesity.

A pathway mediating exercise-induced changes in postprandial metabolism may be the AMPK signalling pathway. AMPK is a kinase with the ability to detect and respond to the energy status of a cell (Towler & Hardie, 2007), in particular promoting energy synthesis when its availability is low. As a relatively new discovery, a plethora of research is available investigating the role for AMPK in metabolism, research which has used both human and animal models. So far, AMPK has been implicated in a number of metabolic pathways, many of which have recently been reviewed by Towler and Hardie (Towler & Hardie, 2007). In muscle, AMPK stimulates glucose uptake and fatty acid oxidation whilst reducing glycogen synthase activity and thus glycogen synthesis, thereby directing glucose towards oxidation and energy production. In the liver, AMPK enhances fatty acid oxidation whilst inhibiting fatty acid and cholesterol synthesis and gluconeogenesis. In adipose tissue, lipolysis and glucose uptake both appear to be inhibited by AMPK. Finally, there is also evidence that AMPK may enhance nitric oxide availability within blood vessels (Towler & Hardie, 2007). Exercise has been reported to stimulate the activity of AMPK (Jorgensen *et al.*, 2006) thus promoting the changes in metabolism mentioned above and interestingly, the activity of AMPK appears to be suppressed by the presence of glycogen (Jorgensen *et al.*, 2006). This may be a mechanism by which glycogen depleting

exercise stimulates AMPK activity and thus alters postprandial metabolism. It might also explain why in a situation where glycogen is more rapidly replenished, for example when the exercise-induced energy deficit is replaced, changes to postprandial metabolism are somewhat attenuated.

Findings from Chapters 3, 6 and 7 suggest that a state of energy balance with high energy turnover induces positive changes in postprandial insulinaemia, energy substrate oxidation and energy substrate balances both immediately and the day following exercise. However, whether such effects persist beyond a single session of exercise remain unknown. A study by Black *et al.* (Black *et al.*, 2005) has, to some extent, addressed this question where six consecutive days of exercise, with the daily energy intake increased to replace the associated energy deficit, were completed prior to metabolic assessment. These authors reported that prolonged exercise when coupled with a state of energy balance had no effect on plasma insulin, glucose or TG concentrations. In their study however, Black *et al.* did not measure the metabolic response to a fat tolerance test and it remains possible that the postprandial responses may still have been attenuated by exercise. Furthermore, they replaced the energy deficit induced by exercise with high carbohydrate drinks and snacks rather than foods of a more balanced composition. The extra carbohydrate provided during the six days is likely to have ensured relatively stable glycogen stores, which as suggested above, may well have mediated the lack of any effect of exercise on insulin concentrations and sensitivity (Wojtaszewski *et al.*, 2002). To address these limitations a similar protocol to that used by Black *et al.* (Black *et al.*, 2005) could be implemented but with the energy deficit replaced using foods of a more balanced composition, foods similar to those used in this thesis. Furthermore, the completion of an oral fat tolerance would provide invaluable insight into the subsequent effects of exercise in a state of energy balance on postprandial metabolism over more prolonged time periods.

8.3 Exercise, energy balance and pulse wave velocity

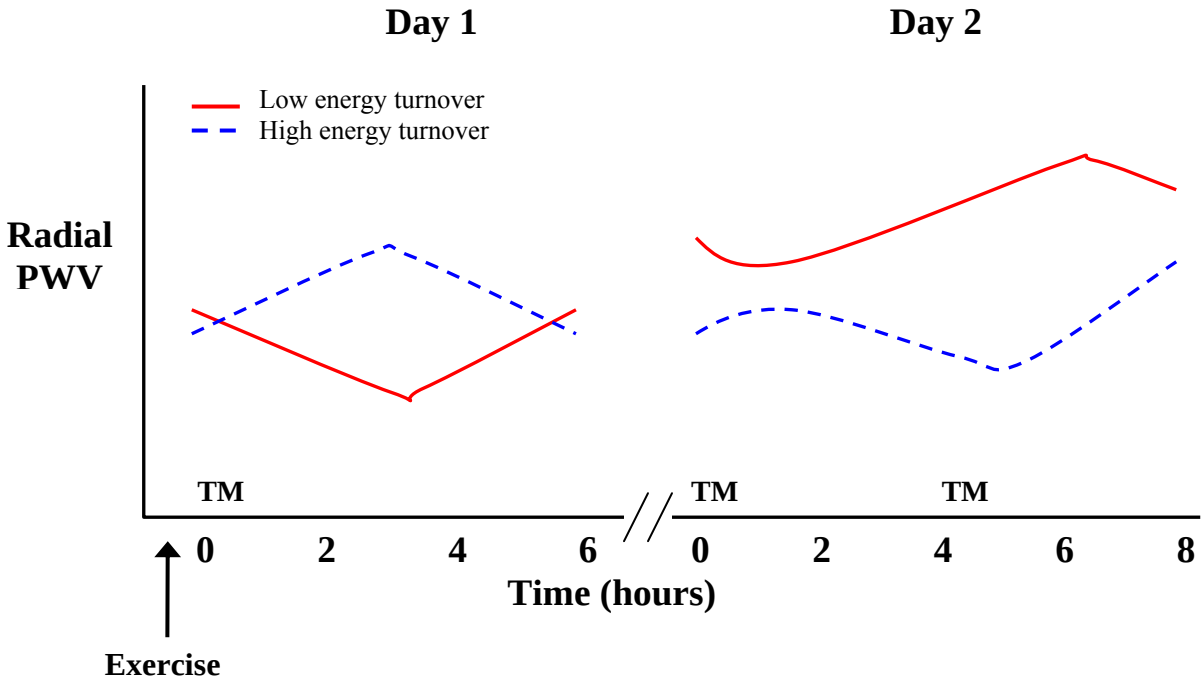


Figure 8.4 Postprandial radial PWV in a state of energy balance with high and low energy turonver.

No differences in postprandial radial or femoral PWV were observed on day one between the different states of energy turnover (**Figure 8.4**). At present there are very little data to compare such findings to, however, a recent study investigated the effects of a single session of exercise performed prior to a test meal on peripheral PWV in young men (Clegg *et al.*, 2007). In contrast to the findings here, they reported peripheral PWV to be lower for up to four hours post exercise, although the lack of any further measurements makes it difficult to determine the true duration of such an effect. One obvious explanation for such different findings is the replacement of the energy expended during exercise in this study, whilst Clegg *et al.* maintained an energy deficit. Another potential difference is subject population as Clegg *et al.* recruited young, healthy active men whilst this study used older, overweight women. The immediate post-exercise decline in fasting radial PWV observed in Chapter 6 is consistent with the findings of others who also reported lower radial PWV up to one hour post exercise (Heffernan *et al.*, 2007b;Naka *et al.*, 2003). A change in femoral PWV previously reported (Heffernan *et al.*, 2007a;Kingwell *et al.*, 1997b) is not however consistent with the lack of any change in fasting femoral PWV reported here. It is possible that again a gender effect might explain the different findings in the immediate response of PWV to exercise; pre-menopausal women were studied in Chapter 6, whereas healthy men were studied by both Kingwell et al. and Heffernan et al. In a healthy group of individuals, reference values for PWV have been shown to be higher in men than

women (Koivistoinen *et al.*, 2007), thus it is possible that individuals with a higher initial PWV may incur greater benefits from exercise. Indeed, in older adults, a population who typically suffer greater levels of impaired endothelial function, an aerobic exercise intervention has been shown to enhance flow mediated dilation to a level comparable to that seen in healthy young men (DeSouza *et al.*, 2000). There are very limited data available investigating the effect of exercise on PWV in women and to the author's knowledge just one has recruited female subjects (Naka *et al.*, 2003) and even then, out of a total of 50 volunteers, just four were women. There is an obvious need for research to recruit both pre-menopausal and post-menopausal women to further investigate whether a gender effect is evident with regard to exercise-induced changes in radial and femoral PWV.

Whilst the immediate response of PWV remains unclear, a novel finding of the present study is that there may be a delayed effect of exercise on lowering peripheral PWV, with significantly attenuated radial PWV observed on day two, in a state of energy balance with high energy turnover (Figure 8.4). The lack of any difference in PWV following exercise with and without energy replacement suggests that lower PWV the day following exercise was independent of the associated energy deficit. Although there is no measure of PWV in the literature to make a direct comparison to, improved endothelial function has been reported the day following 90 minutes of moderate intensity exercise (Gill *et al.*, 2004) and thus it appears that these findings are, to some extent, consistent with the literature. Identifying the regulatory mechanisms for such an effect on PWV the day following exercise is not possible from the data available. Attenuated fasting TG concentrations on day two did appear to mediate lower fasting PWV speeds, an effect which may have driven the lower postprandial PWV observed. Changes in TG have previously been reported to be associated with changes in PWV (Daskalova *et al.*, 2005) and on day one, the lack of any change in TG concentrations with high energy turnover might explain the absence of any change in either postprandial radial of femoral PWV. It is perhaps more likely, however, that the lower radial PWV measured on day two was a result of exercise-induced increases in nitric oxide production and or availability. Nitric oxide, a product of the activity of nitric oxide synthase on L-arginine, is a potent stimulator of vasodilation within a blood vessel (Boger *et al.*, 2003) and has been shown to increase following exercise (Maeda *et al.*, 2004;Roberts *et al.*, 2002). Exercise-induced increases in nitric oxide could promote vasodilation and reduce arterial stiffness, effects which may be reflected in lower PWV. It is possible that lower TG concentrations may have had an indirect effect on PWV via NO. TG particles prevent the uptake and transport of nitric oxide from the blood into

the endothelium of a vessel. Subsequently, nitric oxide is broken down before being able to exert any vasodilatory effect on the blood vessel (Lundman *et al.*, 1997). Lower TG concentrations may therefore facilitate the uptake and transport of nitric oxide into the endothelium, enhancing vasodilation and lowering PWV. Future investigations using a more direct measure of nitric oxide production or availability, such as the nitrate concentration in the blood (Jungersten *et al.*, 1997), may more accurately determine the mechanism by which exercise attenuated radial PWV on day two.

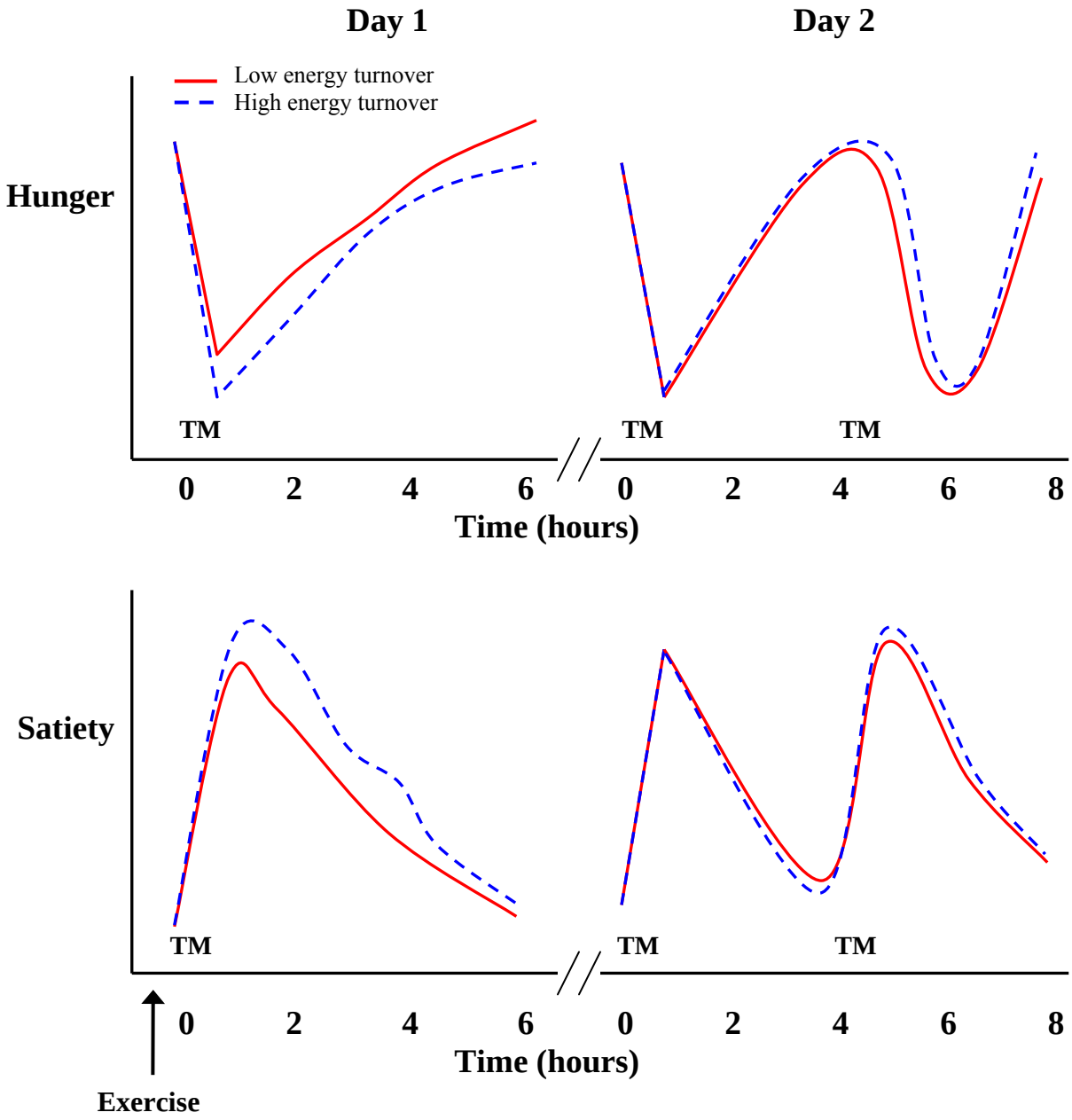


Figure 8.5 Postprandial hunger (top) and satiety (bottom) responses in a state of energy balance with high and low energy turnover.

On day one, hunger appeared to be suppressed and satiety increased in a state of energy balance with high energy turnover (**Figure 8.5**), findings which are consistent with those already available in the literature (King *et al.*, 1994;Tsofliou *et al.*, 2003;Westerterp-Plantenga *et al.*, 1997a) but further show that such an effect of exercise on appetite persists even when a state of energy balance is maintained. The effect of exercise on ratings of hunger and satiety does, however, appear to be transient lasting up to just one hour post-exercise and it is unclear to what extent such short-term changes in appetite will affect long term feeding behaviour and weight regulation.

Resistance to the action of leptin couples obesity (Dyck, 2005) as do elevated circulating concentrations of leptin when compared to normal weight controls (Considine *et al.*, 1996). Higher concentrations of leptin are associated with an increased risk of future CVD (Wallace *et al.*, 2001), therefore it is encouraging that on day two, plasma leptin responses were significantly attenuated in a state of energy balance with high energy turnover compared to low. Although the exercise-induced energy deficit did appear to augment such an effect the difference between the two exercise sessions was not significant. Exercise-induced changes in leptin may have been mediated by changes in energy substrate availability, particularly glucose. Although the mechanism behind such an effect cannot be determined from the data presented here, a possible mechanism is one surrounding the hexosamine biosynthetic pathway, a nutrient sensing pathway that detects glucose flux into cells (Marshall *et al.*, 1991). The end product of this pathway is UDP-*N*-acetylglucosamine, a product which is a know stimuli for leptin secretion (Considine *et al.*, 2000). A reduction in glucose flux along the pathway will reduce UDP-*N*-acetylglucosamine and subsequently restrict leptin production. In Chapter 5, lower insulin concentrations following both exercise trials will likely have restricted glucose uptake into adipocytes and along the hexosamine biosynthetic pathway (Hulver & Houmard, 2003), therefore mediating the lower leptin concentrations observed following exercise both with, and without, energy replacement. The attenuation of the exercise-induced lowering of plasma leptin concentrations observed when the energy expended was replaced, which although was not significant may have been mediated by the additional carbohydrate and therefore glucose provided in the energy replacement meals. Leptin was, however, not measured during the immediate hours following exercise in energy balance and therefore there remains a need for further research to clarify whether exercise in a state of energy balance has any immediate effect on plasma leptin concentrations and to determine the role for glucose in mediating any such exercise-induced changes in plasma leptin.

No effect of energy turnover on total ghrelin concentrations was observed either during day one or day two. This lack of effect is consistent with a number of earlier studies (Burns *et al.*, 2007;Jurimae *et al.*, 2007a;Kraemer *et al.*, 2004;Kyriazis *et al.*, 2007;Martins *et al.*, 2007a) but further shows that total ghrelin is not attenuated by exercise even when the energy deficit is replaced and energy balance maintained. One unexpected finding was that the day following exercise, inverse relationships between total ghrelin, hunger, PFC and desire and positive relationships between total ghrelin, satiety and fullness were observed. Considering the potent orexigenic properties of ghrelin (Druce *et al.*,

2005;Wren *et al.*, 2001), it was expected that the opposite relationships would be seen. Somewhat consistent with these findings is that energy intake at the high energy turnover buffet meal on day one, was inversely correlated with exercise-induced changes in total ghrelin concentrations. Together, these data could suggest that in overweight and obese men and women, exercise in energy balance may alter the normal orexigenic properties of total ghrelin. The author is unclear as to whether this is indeed a true response to exercise and is not able to comment on any particular mechanism that might mediate such an effect. In favour of such a hypothesis is that in response to exercise, overweight and obese individuals fail to show an increase in food intake that is observed in healthy, normal weight controls (Kissileff *et al.*, 1990). However, it is also possible that total ghrelin is in fact not an important mediator of the effect of exercise on appetite and feeding behaviour and instead there is a need to focus on the effects of its individual components, acylated and de-acylated ghrelin

On day one, fasting and postprandial acylated ghrelin concentrations were attenuated in a state of energy balance with high energy turnover, findings consistent with those recently reported by Broom *et al.* (Broom *et al.*, 2007), but also showing the effect to persist in a state of energy balance and not just following exercise with an associated energy deficit. The absence of a separate trial using exercise with an energy deficit does mean that it is not possible at this time to determine whether the effect of exercise on lowering acylated ghrelin concentrations would have been augmented had an energy deficit been maintained, and this is a question that remains to be addressed. It does appear that acylated ghrelin may be an important regulator of appetite and indeed during the hours following exercise the present study reported an inverse relationship between exercise-induced changes in acylated ghrelin and satiety and in their study, Broom *et al.* (Broom *et al.*, 2007) reported a post-exercise suppression in hunger which coincided with lower acylated ghrelin concentrations. It has been suggested that compared to total ghrelin, acylated ghrelin may, to some extent, be regulated by carbohydrate intake; compared to high fat and high protein meals, acylated ghrelin shows a significantly larger attenuation following a meal rich in carbohydrate (Tannous dit *et al.*, 2006) and the consumption of high fat meals has been shown to have no lowering effect on acylated ghrelin concentrations (Al Awar *et al.*, 2005;Tentolouris *et al.*, 2004). Although the direct mechanism by which carbohydrate stimulates a decline in acylated ghrelin is unclear, it is perhaps mediated by the rise in insulin concentrations that accompany greater carbohydrate intakes, and an inverse correlation between insulin and acylated ghrelin has previously been reported (Tannous dit *et al.*, 2006). Although during the immediate post-exercise period no relationship between

insulin and acylated ghrelin was found, there was an inverse relationship between glucose and acylated ghrelin. The author suggests that rather than insulin *per se*, acylated ghrelin might respond instead to insulin mediated glucose uptake although further work is obviously needed to clarify such a relationship. Acylated ghrelin was not measured on day two due to its emergence in the literature during the course of the study. The time course of any exercise-induced attenuation in acylated ghrelin therefore remains unknown. Further investigation might, however, identify a role for exercise-induced changes in acylated ghrelin in mediating appetite and food intake in the days following exercise.

It is interesting that leptin and acylated ghrelin may both, in some way, be mediated by changes in glucose metabolism and availability. Such a mechanism may have important implications when considering the nutritional composition of foods used to replace the energy expended during exercise. If indeed glucose availability regulates leptin and acylated ghrelin responses to exercise, replacing the energy expended with a low or high carbohydrate meal may augment such an effect. However, although high carbohydrate meals may prove beneficial for regulating some hormones, it is important to note that carbohydrate ingestion following exercise attenuates any exercise-induced increase in insulin sensitivity (Cartee *et al.*, 1989). Therefore it may be that the ideal macronutrient composition of the foods used to replace the energy expended during exercise will differ according to the metabolic benefits for which the exercise is targeted i.e. high carbohydrate to attenuate acylated ghrelin but low carbohydrate to preserve insulin sensitivity. Future research may help to determine what the optimal nutritional strategy is when replacing the exercise-induced energy deficit for inducing the maximal metabolic benefits.

The ability of a single session of exercise to successfully modulate energy and macronutrient intake remains inconclusive. In accordance with a number of previous studies, there was no evidence of any change in energy intake or macronutrient intake measured at the buffet meal when in a state of energy balance with either high or low energy turnover, suggesting that in pre-menopausal women, a single session of moderate intensity exercise may not be a sufficient stimulus to induce changes in feeding behaviour. However, it should be noted that energy balance was lower on consumption of the buffet meal after exercise, indicating that there may have been some change in energy intake which failed to reach significance. It is possible that the absence of any obvious change in energy and macronutrient intake may be a reflection of the timing of the buffet meal rather than a true lack of any exercise effect. In Chapter 7, similar to studies already published in the literature (Hubert *et al.*, 1998;Martins *et al.*, 2007a;Pomerleau *et al.*, 2004;Thompson *et*

al., 1988), food intake was measured just a few hours after exercise. It has, however, been suggested that any change in feeding behaviour may occur over a more prolonged period of time. Stubbs *et al.* (Stubbs *et al.*, 2002b) measured food intake in normal weight women during a period of seven days consisting of no exercise, a medium or a high exercise workload. They reported that during the course of the exercise intervention periods, the women significantly increased their energy intake to compensate for approximately 30% of the energy expended during exercise. Additional changes in food intake may also occur beyond a period of days, extending into weeks and maybe month long periods (Blundell *et al.*, 2003). Therefore, it is possible that had energy intake been monitored for a longer period of time after exercise, a change in energy or macronutrient intake might have been observed. Such prolonged measures of food intake should be incorporated, where possible, into future studies

The author made every attempt to ensure that each subject's typical food intake was influenced by as few external factors as possible. To achieve this, subjects were informed that the study was investigating the effects of exercise on food palatability rather than how much they ate. Foods were provided in a manner that avoided any effect of portion size and all foods provided were considered to be palatable by the subjects. All subjects were left to eat alone with the radio, television and magazines all removed to avoid any effect of the media, and the 'social norms' they promote, on food intake. Although such controls were necessary in the laboratory to isolate the effect of exercise and energy balance on subsequent food intake, it should be considered that had the subjects been left to eat the buffet meal in their normal everyday environment, their food intake may actually have differed following exercise. The use of either laboratory or real world protocols both have their strengths and weaknesses and it is difficult to justify the use of one single method all of the time (De Castro, 2000). Therefore, it seems that which method is used should be determined by the research question being investigated. It is however perhaps necessary to confirm findings from the laboratory in a real world setting to determine their relevance for the general population in every day living.

Studies performed in Chapters 3, 4 and 5 investigated the metabolic and behavioural responses to exercise in middle-aged, centrally obese men. Studies performed in Chapters 6 and 7 were completed by overweight and obese pre-menopausal women. Both groups of volunteers were relevant populations to study, especially considering the obesity crisis that the UK currently faces. However, it should also be considered that although highly relevant, the findings of this thesis cannot be directly related to other non-obese

populations. A recent study in men with type 2 diabetes reported 90 minutes of prior exercise to attenuate postprandial insulin concentrations but with no significant effect of exercise on either fasting or postprandial TG or NEFA concentrations, suggesting that in those with type 2 diabetes, the effects of a single session of exercise may differ compared to healthy individuals (Gill *et al.*, 2006a). Furthermore, although the percentage change was the same, the magnitude of the absolute reduction in postprandial TG concentrations and also the reduction in postprandial insulin concentrations has been shown to be greater in obese subjects compared to lean matched controls (Gill *et al.*, 2004). Differences in the appetite response to exercise also make it difficult to directly extrapolate the findings from Chapters 5 and 7 to other populations. In a series of studies, Stubbs *et al*. (Stubbs *et al.*, 2002a;Stubbs *et al.*, 2002b) have shown that women significantly increase their food intake in response to exercise, whilst men failed to show any such compensatory effect. Furthermore, a study in which lean and obese women performed cycling exercise revealed energy consumption at a buffet meal provided a short time after exercise, to be lower in obese women compared with those who were normal weight (Kissileff *et al.*, 1990). Because of the varied metabolic and behavioural responses to exercise observed in different groups of people, there is a need to confirm the findings presented in this thesis both in men and women, the obese and normal weight, the young and old and also in those suffering metabolic diseases such as type 2 diabetes. By doing this, greater insight will be obtained into how a state of energy balance with high energy turnover might regulate postprandial metabolism, appetite control and feeding behaviour and the implications that this may have for the future management and control of cardiovascular disease and obesity.

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APPENDIX 1A

Volunteer Identification Number for this trial:

CONSENT FORM

Title of Project: 'Are the effects of moderate exercise on postprandial metabolism influenced by energy deficit?' OR 'Effects of exercise without energy deficit on subsequent metabolism, substrate utilisation and food satisfaction'.

Name of Researcher:_____

Please initial box

1. I confirm that I have read and understand the information sheet dated 7th April 2006 (version 2) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to take part in the above study. ☐

4. I agree for my samples to be used for future research into the prevention and treatment of obesity and related conditions such as diabetes and heart disease. This may involve analysis of genes associated with these diseases. Yes ☐ No ☐

_____	_____	_____
Name of Volunteer	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent (if different from researcher)	**Date**	**Signature**
_____	_____	_____
Researcher	**Date**	**Signature**

1 for volunteer; 1 for researcher

APPENDIX 1B

HEALTH SCREEN FOR STUDY VOLUNTEERS

(version 2, 25/11/04)

Name: …….

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. At present, do you have any health problem for which you are:

(a) on medication, prescribed or otherwise	yes [ ]	no [ ]
(b) attending your general practitioner	yes [ ]	no [ ]
(c) on a hospital waiting list	yes [ ]	no [ ]

2. In the past two years, have you had any illness which required you to:

(a) consult your GP	yes [ ]	no [ ]
(b) attend a hospital outpatient department	yes [ ]	no [ ]
(c) be admitted to hospital	yes [ ]	no [ ]

3. Have you ever had any of the following:

(a) Convulsions/epilepsy	yes [ ]	no [ ]
(b) Asthma	yes [ ]	no [ ]
(c) Eczema	yes [ ]	no [ ]
(d) Diabetes	yes [ ]	no [ ]
(e) A blood disorder	yes [ ]	no [ ]
(f) Head injury	yes [ ]	no [ ]
(g) Digestive problems	yes [ ]	no [ ]
(h) Hearing problems	yes [ ]	no [ ]
(i) Problems with bones or joints	yes [ ]	no [ ]
(j) Disturbance of balance/co-ordination	yes [ ]	no [ ]
(k) Numbness in hands or feet	yes [ ]	no [ ]
(l) Disturbance of vision	yes [ ]	no [ ]
(m) Thyroid problems	yes [ ]	no [ ]
(n) Kidney or liver problems	yes [ ]	no [ ]
(o) Chest pain or heart problems	yes [ ]	no [ ]
(p) Any other health problems	yes [ ]	no [ ]

4. Have any of your family (parents, grandparents, brothers, sisters, children, aunts, uncles, cousins) ever had any of the following: (if yes please give details including age of first diagnosis)

(a) Any heart problems	yes [ ]	no [ ]
(b) Diabetes	yes [ ]	no [ ]
(c) Stroke	yes [ ]	no [ ]
(d) Any other family illnesses	yes [ ]	no [ ]

4. For females only – Are you postmenopausal?* yes [ ] no [ ]

(*at least 2 years since last menstrual bleeding)

6. Do you currently smoke yes [ ] no [ ]

Have you ever smoked yes [ ] no [ ]

If so, for how long did you smoke and when did you stop? ……

7. How many units of alcohol do you typically drink in a week? ……

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

……

……

……

……

……

……

……

Name and address of GP

……..

……

……

Blood pressure measured at screening……..mm Hg

APPENDIX 1C

PHYSICAL ACTIVITY QUESTIONNAIRE

Name: **Date:**

During one week, how many times on average do you do the following kinds of exercise for more than 15 minutes?

(a) Strenuous Exercise (heart beats rapidly)

For example; running, jogging, squash, vigorous swimming, vigorous long distance cycling.

_____ times per week _____ total minutes per week.

Comments

(b) Moderate Exercise (not exhausting)

For example; fast walking, tennis, easy cycling, badminton, easy swimming, dancing.

_____ times per week _____ total minutes per week.

Comments

(c) Mild Exercise (minimal effort)

For example; yoga, archery, fishing, bowling, golf, easy walking.

_____ times per week _____ total minutes per week.

Comments

APPENDIX 2A

APPETITE QUESTIONNAIRE

Name: _____ **Date:** / / **Trial:** _____

Please answer the following questions by placing a vertical mark through the line for each question.
Regard the end of each line as indicating the most extreme sensation you have ever felt and mark how you feel **NOW.**

Example
This is how to mark this line.

e.g. How happy are you (now)?

Not at all happy _____ As happy as I have ever been

Time: _____

1. How **hungry** do you feel (now)?

I am not hungry at all _____ I have never been more hungry

2. How **satisfied** do you feel (now)?

I am completely empty _____ I cannot eat another bite

3. How **full** do you feel (now)?

Not at all full _____ Totally full

4. How **much** do you think you **can eat** (now)?

Nothing at all _____ A lot

5. How strong is your **desire to eat** (now)?

Not at all strong _____ Very strong

6. Would you like to **eat something sweet** (now)?

Yes, very much _____ Not, at all

7. Would you like to **eat something salty** (now)?

Yes, very much _____ Not, at all

8. Would you like to **eat something savoury** (now)?

Yes, very much _____ Not, at all

9. Would you like to **eat something fatty** (now)?

Yes, very much _____ Not, at all

APPENDIX 2B

PALATABILITY QUESTIONNAIRE

Name: **Date:** / / **Trial:**

Please answer the following questions by placing a vertical mark through the line for each question.
Regard the end of each line as indicating the most extreme sensation you have ever felt and mark how you feel **NOW.**

1. How **appealing** does your meal look?

Good _____ Bad

2. How does your meal **smell**?

Good _____ Bad

3. How does your meal **taste**?

Good _____ Bad

4. How much of an **aftertaste** has your meal left?

Much _____ None

5. How **palatable** have you found the meal?

Good _____ Bad

APPENDIX 3A

DIETARY QUESTIONNAIRE

The information you give me in this qustionnaire will help me to plan your diets for the 3 days prior to each trial. Where it is relevant, please give me as much dietary information as possible so that I can make your meals as nice as possible.

1) What cereal do you prefer?

- Frosties ☐
- Weetabix ☐ Sugar ☐ tsp
- Crunchy nut cornflakes ☐

2) What milk do you drink?

- Skimmed ☐
- Semi-Skimmed ☐
- Full ☐

3) What bread do you prefer?

- White / Wholemeal

4) What do you prefer in your sandwiches?

- Cheese ☐
- Ham ☐
- Tuna & Mayonnaise ☐

5) What flavour crisp do you prefer?

- Salt & vinegar ☐
- Cheese & onion ☐
- Plain ☐
- Prawn cocktail ☐

6) Do you drink tea?

- If yes how many cups do you have in one day?

- Do you take milk in your tea? **Yes** **No**

- How many teaspoons of sugar do you take in your tea?

7) Do you drink coffee?

- If yes how many cups do you have in one day?

- Do you take milk in your coffee? **Yes** **No**

- How many heaped teaspoons of sugar do you take in your coffee?

8) Please list any foods that you do not like?

9) Do you have any specific food requirements i.e. vegetarian?

10) Do you have any food allergies? If yes please list all of them clearly in the space below.

APPENDIX 3B

FOOD INVENTORY INSTRUCTIONS

It is important that you weigh and record everything that you eat and drink over a three-day period. This information will be used when I am preparing your diet for the days prior to each trial. Please include a weekend day as one of the three. You have been given weighing scales and a food inventory record sheet to make this easier for you.

Please (i) start a separate page for each day.
(ii) start a separate line for each item.

Column 1
Record meal and time and place of eating.

Column 2
Describe each item as accurately as possible, stating where relevant:
(i) type and brand
(ii) whether food is fresh, dried, canned, frozen, salted, smoked, etc.
(iii) whether food is cooked, if so give cooking method e.g. fried, baked, etc.

Column 3
Record the weight of each item after cooking:
(i) place scales on a level surface
(ii) place plate or container on top of scales
(iii) press 'ON/Reset' button to turn on scales
(iv) once zero appears, add first item of food
(v) record weight displayed

Wherever possible, record weights in grams. If this is not possible, record weights in household measures (e.g. sugar or jam in teaspoons, stating whether level, rounded, or heaped).

Column 4
Record the weight of any leftovers, such as food remaining on plate, weight of container in which food has been weighed, apple cores, etc.

Columns 5 and 6
Please leave blank.

If food consists of several items, please list each on a separate line i.e. instead of writing 'one cheese sandwich', record separately the weights of bread margarine, cheese, etc.

Please remember to record all drinks, as well as food, giving weights where possible, or volumes if these are known. Record separately the weights of added milk and sugar.

An example is shown overleaf.

Food Inventory – Example

Name_____ Date _____

1. Time/Place	2. Description of food/drink	3. Weight food/drink (g)	4. Weight container/ leftovers (g)	Leave Blank	
Breakfast	Cornflakes (Kelloggs)	28			
8:30am	Milk (Sainsbury's virtually fat-free)	48			
Home	Bread (Mothers Pride, large white	76			
	sliced, toasted)				
	Flora margarine	7			
	Robinsons lemon marmalade	12			
	Coffee (instant)	2			
	Milk (whole pasteurised)	10			
Lunch	Cheese (Cheddar)	55			
1:00pm	Bread (white, crusty)	76			
Pub	Butter	4			
	Chutney (2 teaspoons)				
Snack	Coffee (instant)	2			
3:30pm	Coffee-mate	6			
Office	Mars Bar	35			
	Apple	76	8 (core)		
Dinner	Turkey Fillet (frozen, grilled)	102			
6:30pm	Potatoes, old, boiled	320	74		
Home			(leftover)		
	Peas (Birds Eye, frozen, boiled)	50			
	Heinz tomato ketchup	14			
	Yoghurt (Ski strawberry thick and	162	10		
	creamy)		(carton)		
	Coffee, filter	148			
	Milk (Sainsbury's virtually fat-free)	8			
Snack	Banana	107			
7:45pm	Orange Tango (can)	330			
Home					

Food Inventory

Name_____ Date _____

1. Time/Place	2. Description of food/drink	3. Weight food/drink (g)	4. Weight container/ leftovers (g)	Leave Blank	

Food Inventory

Name_____ Date _____

1. Time/Place	2. Description of food/drink	3. Weight food/drink (g)	4. Weight container/ leftovers (g)	Leave Blank	

Food Inventory

Name_____ Date _____

1. Time/Place	2. Description of food/drink	3. Weight food/drink (g)	4. Weight container/ leftovers (g)	Leave Blank	

APPENDIX 4A: Insulin

Reagents

1 x 96 well coated plate

Calibrators: 3, 10, 30, 100 and 200 $mU.l^{-1}$

Calibrator 0

Enzyme conjugate

Enzyme conjugate buffer

Wash buffer

Substrate TMB

Stop solution

Reagent preparation

Wash buffer: the concentrated wash buffer was diluted with distilled water by adding 35 ml of buffer to 700 ml distilled water.

Enzyme conjugate: the enzyme conjugate was diluted with enzyme conjugate buffer by adding 1.0 ml of the enzyme conjugate to 10 ml enzyme conjugate buffer.

Procedure

i. 25 µl aliquots of calibrators and plasma samples were added into the appropriate wells.
ii. 100 µl of enzyme conjugate was added into each well.
iii. The plate was incubated for 1 hour at room temperature on a plate shaker.
iv. The plate was washed using an automated plate washer, with 350 µl of wash buffer added to and then aspirated from each well a total of six times.
v. 200 µl TMB was added into each well before being incubated in the dark, at room temperature for 15 minutes
vi. 50 µl stop solution was added into each well and the plate lightly shaken for 5 seconds
vii. The plate was read at an optical density of 450 nm.

APPENDIX 4B: Total ghrelin

Reagents

20 x assay buffer concentrate

96 well immunoplate

Primary antiserum

Standard peptide

Biotynylated peptide

Streptavidin-horseradish peroxidase (SA-HRP)

Substrate TMB

2N HCl

Reagent preparation

Buffer solution: The 20x assay buffer was diluted with 950 ml distilled water

Stock solution: The standard peptide was rehydrated with 1.0 ml of buffer solution and allowed to stand for 10 minutes

Standard peptide solutions: No.1 was prepared by adding 100 µl of stock to 900 µl buffer solution, No.2 by adding 100 µl of No.1 to 900 µl buffer solution, No.3 by adding 100 µl of No.2 to 900 µl buffer solution, No.4 by adding 100 µl of No.3 to 900 µl buffer solution and No.5 by adding 100 µl of No.4 to 900 µl buffer solution

Primary antiserum: Rehydrated with 5.0ml of buffer solution

Biotynylated peptide: Rehydrated with 5.0ml buffer solution

SA-HRP: 12 µl SA-HRP was added into 12 ml of buffer solution and mixed thoroughly

Procedure

i. Leaving wells A1 and A2 blank, 50 µl of buffer solution was added into B1 and B2 and 50 µl of peptide standard No.1 to No.5 from C1 and C2 through to G1 and G2
ii. 50 µl of sample were added into the appropriate wells
iii. 25 µl of primary antiserum was added into every well except the blank
iv. 25 µl of biotynylated peptide was added into every well except the blank
v. The plate was incubated for 2 hours at room temperature on an orbital shaker
vi. Each well was manually washed by adding 350 µl buffer solution and blotted dry 4 times.
vii. 100 µl of SA-HRP was added into each well and then incubated for 1 hour, at room temperature on an orbital shaker.

viii. Each well was re-washed as in vii

ix. 100 µl of TMB was added into each well and then incubated, in the dark, for 1 hour at room temperature

x. 100 µl 2N HCl was added into each well to stop the reaction and the plate was read, within 20 minutes, at an optical density of 450 nm.

APPENDIX 4C: Acylated ghrelin

Reagents

96 well immunoplate

Anti-acylated ghrelin tracer

Human acylated ghrelin standard

Quality control sample

EIA buffer

Concentrated wash buffer

Tween 20

Ellman's reagent

Reagent preparation

EIA buffer: 50 ml of distilled water was added to the vial of EIA buffer, dissolved and mixed

Human acylated ghrelin standard: 1.0 ml of distilled water was added to the vial and left to dissolve making standard 1 (S1). S2 was made by adding 500 µl of S1 to 500 µl EIA buffer, S3 by adding 500 µl of S2 into 500 µl EIA buffer, S4 by adding 500 µl of S3 into 500 µl EIA buffer, S5 by adding 500 µl of S4 into 500 µl EIA buffer, S6 by adding 500 µl of S5 into 500 µl EIA buffer, S7 by adding 500 µl of S6 into 500 µl EIA buffer and S8 by adding 500 µl of S7 into 500 µl EIA buffer

Quality control: 1.0 ml of distilled water was added to the vial, dissolved and mixed

Anti-acylated ghrelin-AchE tracer: 10 ml of EIA buffer was added to the vial, dissolved and mixed

Wash buffer: 1.0 ml of concentrated wash buffer was diluted to 400 ml with distilled water. 200 µl of tween 20 was added and mixed

Ellmans reagent: Prepared five minutes before use. 49 ml of distilled water was added to the vial along with 1.0 ml of concentrated wash buffer. Contents were mixed thoroughly

Procedure

i. 100 µl of EIA buffer was added into wells A2 to H2
ii. 100 µl of each of the eight human acylated ghrelin standards were added into wells A3 to H4.
iii. 100 µl of the quality control and samples were added into the appropriate wells

iv. 100 µl of the anti-acylated ghrelin-AChE tracer was added into each well except the blanks (A1 to H1).
v. The plate was incubated overnight for 20 hours at 4°C, after which, each well was washed 10 times with 300 µl of wash buffer.
vi. 200 µl of Ellman's reagent was added into every well and the plate was incubated, in the dark for 30 minutes on a plate shaker.
vii. The plate was read at an optical density between 405 and 414

APPENDIX 4D: Assymetric Dimethylarginine (ADMA)

Reagents

12 Microtiter strips

Standards 0, 0.1, 0.3, 0.6, 1.0 and 5.0 $\mu mol.l^{-1}$

Controls 1 and 2

Acylation Buffer

Acylation Reagent

Antiserum

Enzyme Conjugate

Wash Buffer

Substrate TMB

Stop Solution

96 well Reaction plate

Equalising Reagent

Dimethylformamide (DMF)

Reagent preparation

Wash Buffer: The contents were diluted with distilled water to make 500 ml of solution

Equalising Reagent: 5.0 ml of distilled water was added to the vial, mixed and then left on a roll mixer for 30 minutes.

Acylation Reagent: The contents of one bottle were dissolved with 1.5 ml DMF and shaken for five minutes using an orbital shaker. The Acylation Reagent was prepared immediately prior to use.

Procedure for stage one: Acylation

i. 20 µl each of standards, controls 1 and 2 and plasma samples were pipetted into the appropriate wells on the Reaction Plate
ii. 25 µl of Acylation Buffer and then 25 µl of Equalising Reagent were added into each well
iii. The plate was mixed for 10 seconds using an orbital shaker
iv. 25 µl of Acylation Reagent was added into each well and mixed immediately
v. The plate was incubated on an orbital shaker, at room temperature, for 30 minutes
vi. 1.5 ml of the prepared Equalising Reagent was diluted in 9.0 ml distilled water and mixed. 100 µl of this solution was added into each well
vii. The plate was incubated on an orbital shaker, at room temperature, for 45 minutes

Procedure for stage two: The ELISA

i. Any small fibrinogen like clot that had developed in the wells was removed
ii. 50 µl of the prepared standards, controls and samples on the reaction plate were pipetted into the respective wells of the microtiter strips
iii. 50 µl of antiserum was added to each well and briefly shaken on an orbital shaker.
iv. The plate was covered and incubated for 15-20 hours overnight at 2-8°C
v. Each well was washed using 250 µl of Wash Buffer four times
vi. 100 µl of enzyme conjugate was added into each well and incubated for 60 minutes on an orbital shaker at room temperature
vii. Each well was washed using 250 µl of Wash Buffer four times
viii. 100 µl of Substrate TMB was added into each well and incubated, in the dark and at room temperature, for 20 to 30 minutes on an orbital shaker
ix. 100 µl of Stop Solution was added into each well and the plate was read at an optical density of 450 nm

APPENDIX 4E: Triglyceride (TG)

Principle of method

A lipoprotein lipase derived from micro-organisms is used to rapidly and completely hydrolyse TG to glycerol followed by the oxidation of glycerol to dihydoxylacetone phosphate and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminophenazone and 4-chlorophenal under the catalytic action of peroxidase to form a red dyestuff.

$$\text{Triglycerides} + 3\ H_2O \xrightarrow{\text{LPL}} \text{glycerol} + 3\ \text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 2\ H_2O + HCl$$

Reagents

R1 Buffer / 4-chlorophenol / enzymes

Calibrator S1: 0.9% NaCl

Calibrator S2

Quality Control Sera

Procedure

Plasma TG concentrations were determined by members of staff in the Clinical Biochemistry Department at Glasgow Royal Infirmary. The procedure for such analysis is property of this department.

APPENDIX 4F: Non-esterified fatty acids (NEFA)

Principle of method

$$\text{RCOOH (NEFA)} + \text{ATP} + \text{CoA-SH} \xrightarrow{\text{ACS}} \text{Acyl-CoA} + \text{AMP} + \text{Ppi}$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{ACOD}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminophenazone} + \text{MEHA} \xrightarrow{\text{POD}} \text{Quinonelmine-colour} + 4\ H_2O$$

The intensity of the red colour the reaction produces is equivalent to the concentration of fatty acids in the plasma sample.

Reagents

R1: Solvent A

R1a: Colour Reagent A

R2: Solvent B

R2a: Colour Reagent B

CAL: NEFA C Standard

Reagent preparation

Colour Reagent Solution A: The contents of R1a were dissolved with 10 ml of R1 and mixed well

Colour Reagent Solution B: The contents of R2a were dissolved with 20 ml of R2 and mixed well

Procedure

Plasma NEFA concentrations were determined by members of staff in the Clinical Biochemistry Department at Glasgow Royal Infirmary. The procedure for such analysis is property of this department.

APPENDIX 4G: Glucose

Principle of method

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^{+} \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^{+}$$

Reagents

R1: Buffer / Coenzymes

R2: Diluent

2. Reagent

Reagent preparation

R2: Reagent 2 was added to R2

Procedure

Plasma glucose concentrations were determined by members of staff in the Clinical Biochemistry Department at Glasgow Royal Infirmary. The procedure for such analysis is property of this department.

APPENDIX 4H: Total Cholesterol

Principle of method

The analysis of total cholesterol is based on the determination of Δ^4-cholestenone after enzymatic cleavage of the cholesterol ester by cholesterol esterase. Cholesterol is then converted by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxidise, subsequently producing red dyestuff, the intensity of which is proportional to the concentration of cholesterol in the sample.

$$\text{Cholesterol esters} + H_2O \xrightarrow[\text{esterase}]{\text{Cholesterol}} \text{cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{Cholesterol}} \text{cholest-4-en-3-one} + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 4\ H_2O$$

Reagents

R1 Cholesterol reagent

S1 0.9% NaCl

S2 Calibrator for automated systems

0.9% NaCl

Procedure

Plasma total cholesterol concentrations were determined by members of staff in the Clinical Biochemistry Department at Glasgow Royal Infirmary. The procedure for such analysis is property of this department.

APPENDIX 4I: High Density Lipoprotein (HDL) Cholesterol

Principle of method

This method for measuring HDL cholesterol uses polyethylene glycol (PEG)-modified enzymes and dextran sulphate. When PEG modifies cholesterolesterase and cholesterol oxidase enzymes, they display selective catalytic activities towards lipoprotein fractions, the activity increasing in order: LDL < VLDL ≈ chylomicrons < HDL.

In the presence of magnesium sulphate, dextran sulphate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG modified enzymes. At the start of the reaction, with the addition of R2, the cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups. Cholesterol esters are broken down into free cholesterol and fatty acids and in the presence of oxygen, cholesterol is oxidised to form Δ^4-cholestenone and hydrogen peroxide. The presence of peroxidise ultimately forms a blue-purple pigment, the intensity of which is proportional to the cholesterol concentration.

$$\text{HDL-cholesterol esters} + H_2O \xrightarrow[\text{esterase}]{\text{PEG-cholesterol}} \text{cholesterol} + \text{RCOOH}$$

$$\text{HDL-cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{PEG-cholesterol}} \Delta^4\text{-cholestenone} + H_2O_2$$

$$2\ H_2O_2 + \text{4-amino-antipyrine} + \text{HSDA} + H^+ + H_2O \xrightarrow{\text{Peroxidase}} \text{Purple-blue pigment} + 5\ H_2O$$

Reagents

R1 Dextran Sulfate / buffer

R2 PEG-enzymes / 4-amino-antipyrine / buffer

Procedure

Plasma HDL cholesterol concentrations were determined by members of staff in the Clinical Biochemistry Department at Glasgow Royal Infirmary. The procedure for such analysis is property of this department.

APPENDIX 4J: 3-hydroxybutyrate (3-OHB)

Principle of method

The measurement of 3-OHB is based on the breakdown of 3-OHB to acetoacetate via the action of the enzyme 3-hydroxybutyrate dehydrogenase. In parallel with this reaction, NAD^+ is reduced to NADH and it is the associated change in absorbance associated with such a reaction that is correlated with the concentrations of 3-OHB in the plasma sample.

$$3\text{-OHB} + NAD^+ \xrightarrow{\text{3-hydroxybutyrate dehydrogenase}} \text{acetoacetate} + H^+ + NADH$$

Reagents

Buffer

Enzyme / Coenzyme

Standard

Procedure

i. The following volumes of the standard or sample, distilled water and Reagent were pipetted into individual curvette segments

	Micro		**Semi Micro**	
	Standard / sample	**Reagent Blank**	**Standard / sample**	**Reagent Blank**
Standard / sample	75 µl	/	25 µl	/
Distilled water	/	75 µl	/	25 µl
Reagent	3.00 ml	3.00 ml	1.00 ml	1.00 ml

ii. All liquids were mixed before being incubated for one minute at 37°C and the first reading was taken.

iii. The absorbance was then read again after one and two minute intervals and the mean absorbance change, per minute, was used in the calculation of 3-OHB concentrations.

APPENDIX 4K: Leptin

Principle of method

A fixed concentration of a labelled tracer is incubated with a constant dilution of antiserum. In this situation, the concentration of antigen binding sites is limited. The addition of unlabelled antigen introduces competition between the labelled tracer and unlabelled antigen for the limited number of antibody binding sites. Therefore, as the concentrations of unlabelled antigen increases, the amount of tracer attached to the antibody will decrease. This process is measured by separating the bound antibody from the free tracer and counting either or both of the fractions.

Reagents & Procedure

i. Radioiodinated leptin was prepared using a solid phase lactoperoxidase procedure.
ii. Leptin was purified by Sephadex G-25 gel filtration followed by G-50 using 0.1 $mol.l^{-1}$ phosphate, 7.7 mmol sodium $azide.l^{-1}$, $1g.l^{-1}$ BSA and 0.5 $ml.l^{-1}$ Triton X-100 elution buffer.
iii. 0.1 ml of test plasma was incubated with the leptin standard, 0.1 ml donkey serum, 0.1 ml assay buffer, shhep antileptin antiserum and 125I-labelled leptin at 4˚C for 16 hours.
iv. Sepharose-donkey antisheep globulin was added after incubation and the samples were then re-incubated for one hour at room temperature.
v. The free and bound fractions were separated by centrifugation using three 3-ml washes with 0.15 $mmol.l^{-1}$ sodium chloride containing Tween 20.
vi. The bound fraction was counted for 60 seconds on a multichannel counter.